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Tissue localization of some teratogens at early and late gestation related to fetal effects

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TISSUE LOCALIZATION OF SOME TERATOGENS AT EARLY AND LATE GESTATION RELATED TO FETAL EFFECTS

by

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TO ANNELI, JAKOB AND MARKUS

INTRODUCTION

The factors affecting fetal development and malformed babies have always interested man. The reader interested in the historical aspects in this field is referred to e.g. Ferreira (1969) or Warkany (1972).

After the acceptance of the Mendelian laws, "congenital" and "hereditary" often became synonymous concepts when malformations were considered. However, already about a 100 years ago chemicals introduced into the mother were found to pass to the fetus, e.g. salicylic acid and chloroform, which were correlated to icterus neonatorum (Zweifel 1877). Before the thalidomide catastrophe in the early sixties only a few drugs (e.g. antineoplastic and progestational agents) were known to produce real malformations of the human fetus. It has been possible to cause malformations in animal experiments by administration of large doses of a great number of chemicals. Under the conditions of human usage only some of them have proven to cause fetal damage at this time. Of these the vaginal carcinomas recently reported in young women whose mothers took synthetic estrogens (stilbestrol) during early pregnancy (Herbst et al. 1974) perhaps deserve special concern. More detailed information on the risks for human fetal damage can be found in publications by e.g. Kelsey (1965), Shephard (1973) and Wilson (1973). Wilson (1973) has calculated that the cause of about two thirds of all developmental defects in man is unknown. It is often suggested that a significant portion results from an interaction between e.g. external chemicals and a genetic disposition.

Our knowledge concerning the mechanisms by which chemicals cause fetal damage is very sparse, and only in a few instances have generally accepted theories been presented. Most often there is no specific effect for a given chemical; rather it seems that one agent produces a wide spectrum of malformations - which indicates a nonspecific mechanism of action. Moreover, different chemicals may often produce the same type of malformation (Saxén & Rapola 1969). This confusing aspect may partly be explained by the fact that the different organs have certain sensitive periods in their development, when they are especially susceptible to external influences (Brent 1960; Degenhardt

& Kleinebrecht 1971).

The question concerning the degree to which a teratogenic agent passes from the mother to the fetus is an essential part of the complex picture of teratogenic mechanisms. The laws regulating transplacental passage (in late gestation) have been dealt with in many publications including review articles (Flexner & Gellhorn 1942; Page 1957; Widdas 1961; Moya & Thorndike 1962; Longo 1972; Maickel & Snodgrass 1973; Mirkin 1973; Nishimura 1973; Ullberg 1973).

These laws, however, can probably not be applied to the early postimplantation period without modifications, as the anatomical situation is quite different at that time, both in man and experimental animals. In fact, there is little information available on the uptake of foreign substances as well as nutrients in the embryonic tissues. This situation certainly is due to the difficulties in collecting young embryos. One approach to the problem has been tried mainly by Lutwak-Mann and her co-workers (Lutwak-Mann et al. 1960; Lutwak-Mann 1962; Lutwak-Mann & McIntosh 1971; McIntosh & Lutwak-Mann 1972; Lutwak-Mann 1973), but also by other groups (see e.g. Farbo 1973). In these studies free-floating (preimplantation) blastocysts, or blastocoelic fluid after implantation, have been collected after administration of nutrients or foreign compounds and have been analysed. Although of fundamental value for the understanding of reproductive physiology, these studies have not given answers to the question of accumulation of chemicals in the embryonic cells in the early organogenetic phase.

The technique of whole body autoradiography has contributed to our knowledge concerning the degree to which drugs and other chemicals pass the placental barrier and localize in certain fetal tissues. Some findings were reviewed by Ullberg (1973) and Dencker & Ullberg (1973). The distribution pattern within a fetus most often shows similarities with the maternal pattern but is generally more even. The fetus seems to lack some transport and accumulation mechanisms which are present in the adult animal e.g. in the endocrine and excretory organs.

A frequent finding is a strong and selective accumulation in one single type of fetal tissue. This may involve a more serious risk for damage to the fetus than a corresponding accumulation in the mother does to the mother, since the fetal organs are in a sensitive developmental phase.

Among these specific localizations some may be mentioned: the localization of tetracycline in the bone structures (André 1956;

Blomquist & Hanngren 1966); thiouracil in the thyroid (Slanina et al. 1973); a number of polycyclic compounds (e.g. chloroquine and chlorpromazine) in the melanin of the eye and inner ear (Lindquist & Ullberg 1972; Lindquist 1973; Dencker & Lindquist 1975; Dencker et al. 1975). These localizations can be correlated to reported organ damage in human fetuses after the mothers had taken these respective drugs during pregnancy.

The autoradiographic investigations so far have been done almost exclusively in late gestation. The few investigators who have tried to localize chemicals in early pregnancy by means of autoradiography have used histological techniques that visualize only substances firmly bound to the tissues (e.g. nucleic acids, amino acids). To my knowledge no reports have appeared where techniques which avoid the dislocation of soluble isotopes have been used for the systematic investigation of this problem.

The aim of this study has been to use the principles of the whole body autoradiography technique to obtain as much information as possible concerning tissue localization of teratogens in embryos and the surrounding membranes and placental structures without redistributing the chemical to be investigated. Instead of using the whole animals, the uteri have most often been removed and placed on a horizontal plate on a microtome stage to be sectioned. This allowed the taking of thin and even sections. All embryos could be represented by the same section and all embryonal and placental structures could be seen and identified if many sections were taken. In late pregnancy when the fetuses were larger, the whole pregnant animal could be easily investigated. At this stage the relations between the fetal and the maternal distribution patterns were of interest.

An investigation like that outlined above may hopefully throw some light upon the following questions:

1. Are there differences in the uptake of drugs and chemicals in the embryo-fetus at the different stages of gestation?

2. Is it possible to determine the route of transfer to the embryo and fetus in the different stages (i.e. in relation to the varying function of the placental structures)?

3. Is there at all an embryonic uptake or may teratogenic effects be explained by extraembryonic mechanisms?

4. If there is an embryonic uptake, may changes in the teratogenic sensitivity of a compound be explained by a similar change in the accumulation in the embryo or fetus?

When so different problems are dealt with the choice of substances to be studied creates some problems. All of the substances chosen in the present study are of interest in relation to the teratogenic effects in the animals used. *Cadmium*, *trypan blue* and *inorganic mercury* (chapters I-III) are considered essentially not to be transferred to the fetus in late gestation but are teratogenic in early gestation. The two organic acids, *2,4,5-trichlorophenoxyacetic acid* (2,4,5-T) and *salicylic acid*, on the other hand, reach relatively high concentrations in late gestation. They have very different effects on the embryo and have different periods when embryonic damage occurs (chapters IV and V). Finally the metal *zinc*, which seems to play a physiological role in embryonic development has been studied (chapter VI). All these substances with the exception of trypan blue have been autoradiographed. Mercury, 2,4,5-T and salicylic acid have also been measured by impulse counting. Trypan blue has been identified in the unstained sections by its colour.

There may possibly be sources of misinterpretations in the nomenclature of this monograph. One of these is the manner of determining the pregnancy stage. The day following the night of conception is consistently called day 1 or the first day of gestation. It means that e.g. an injection in a mouse made at 12.00 hours on day 8 is made 7 days 12 hours after conception (as this is supposed to occur at midnight). When a more exact timing is desired, the example 7 days 12 hours is used, otherwise only day 8 or the 8th day of gestation. Difficulties also arise when a comparison is made of the present timing with other timings from the literature, but when possible they have been adjusted.

In the literature the expression "yolk sac" is often inappropriately used denoting the visceral yolk sac endoderm. In this study the yolk sac cavity, the visceral yolk sac (endoderm) (sometimes called yolk sac placenta), and the parietal yolk sac (endoderm) are the terms used. When "placenta" or "placental labyrinth" are used alone it refers to the chorioallantoic placenta. The reader not well oriented in the anatomy of embryonic-fetal and placental development is referred to chapter VII or to Rugh (1968) and Langman (1969) for descriptions of mouse and human development, respectively.

One report of a part of chapter I has appeared earlier (Dencker 1975).

CHAPTER I

CADMIUM: ACCUMULATION IN THE EMBRYOS, PLACENTAE AND OVARIES RELATED TO EFFECTS ON REPRODUCTION

The biological effects of cadmium deserve attention for many reasons. Its use in different industrial processes and products has increased its presence in the environment. Its exceptionally long half-life in the human body, 10 to 30 years (Friberg et al. 1974), stresses the importance of a better understanding of its action. Cadmium exerts specific effects in the body, e.g. on the steroid producing organs (Pařizek 1957; Kar et al. 1959; Kaul & Ramaswami 1970). In late gestation it causes placental necrosis, especially in the pars fetalis, and subsequent fetal death (Pařizek 1964). In the mother, it provokes pathological changes with clinical and morphological similarities to human toxemia of pregnancy (Pařizek 1965).

In the golden hamster during early gestation (8th to 9th day), cadmium causes resorptions and embryonic structural defects, the character of which is dependent on the time of administration (Ferm & Carpenter 1967; Ferm 1971; Gale & Ferm 1973). These authors thought that cadmium was an embryonic "site-specific teratogen" due to its interference with specific enzymatic reactions. In the rat (Scharpf et al. 1972; Barr 1973) and in the mouse (Schroeder & Mitchener 1971), malformations and fetal death are produced by cadmium.

A whole body autoradiographic study in the mouse during late gestation revealed no fetal uptake of radioactive cadmium (Berlin & Ullberg 1963 b), but Ferm et al. (1969) showed that small amounts of radioactive cadmium reached the fetus at the time of embryogenesis (8th to 9th day) in the golden hamster. Lucis et al. (1971) showed that small amounts of cadmium passed the placenta during the first third of gestation in the rat, and Chaube et al. (1973) found hardly detectable quantities in human fetuses of different ages.

This investigation was undertaken to study the possible accumulation of cadmium in embryonic (fetal) and placental structures and also in the whole maternal genital apparatus in mice and golden hamsters at different stages of gestation.

Isotope

Carrier free $^{109}CdCl_2$ (spec. act. 95 and 280 μCi/μg respectively) was obtained from The Radiochemical Centre, Amersham, England, dissolved in 0.1 M-hydrochloric acid (2 μg ^{109}Cd/ml). This was diluted with distilled water so that 0.053 and 0.017 M-hydrochloric acid contained 100 μCi (1.05 and 0.36 μg respectively) ^{109}Cd/ml before injection.

Animals

Three months old golden hamsters (*Mesocricetus auratus*), weighing about 110 g, and mice (C57BL and NMRI), weighing about 25 g, were used in this study. The animals were maintained on a complete pellet diet (Anticimex, Sollentuna or AB Ewos, Södertälje, Sweden) at room temperature (25°C) and were allowed free access to water. The dark period was during the night and lasted from 18.00 to 6.00 hours.

The hamsters were mated between 19.00 and 21.00 hours, and the next day was designated day 1 of gestation. The mice were mated overnight. The presence of a vaginal plug on the following morning was taken to indicate conception, and this day was similarly called day 1. Before administration of ^{109}Cd the pregnancy was confirmed by palpation through the abdominal wall, which could be done in the hamster only after light ether anesthesia.

At predetermined pregnancy stages the animals received an intravenous injection, the hamsters into a femoral or jugular vein under light ether anesthesia, and the mice into a tail vein (without anesthesia). The hamsters received 20 μCi (0.21 or 0.072 μg) and the mice 5 or 10 μCi (0.04, 0.05 or 0.11 μg) each. The injection schedule showing gestational ages at the time of injection, postinjection survival intervals and the main findings can be found in table I:1. The gestational age was determined by the assumption that fertilization occurred on an average at 24.00 hours in the mice and 20.00 hours in the hamster. As all the different doses were tracer doses compared to the teratogenic ones, the dose of the individual animal is not shown in the table. It may be noted that the mouse with 53 days survival time was injected 35 days prior to mating.

After the different survival times, the animals were killed by prolonged ether anesthesia. The abdomen was opened and the uterus,

TABLE I:1. Injection regimen of $^{109}CdCl_2$ into pregnant golden hamsters and mice and the main subsequent distribution in the embryos or fetuses and in the placental structures.

Day of gestation on which ^{109}Cd injected	*Interval between conception and injection*		*Interval between injection and autopsy*	*Main area of uptake*
	Days	*Hours*		
Hamster				
8	7	14	4 hr	Embryonic gut
8	7	14	24 hr	Embryonic gut
8	7	14	4 days	Embryonic gut
8	7	20	24 hr	Embryonic gut
9	8	12	8 hr	Embryonic gut
9	8	17	4 hr	No embryonic uptake
9	8	17	8 hr	Embryonic gut
9	8	21	16 hr	No embryonic uptake
9	9	2	16 hr	No embryonic uptake
Mouse				
8 (NMRI)	7	12	24 hr	Embryonic gut
8 (NMRI)	7	12	3 days	Traces in the gut
8 (NMRI)	7	12	5,7,9 and 11 days	No embryonic uptake
8 (C57BL)	7	22	12 hr	Embryonic gut
9 (C57BL)	8	13	8 hr	Embryonic gut
9 (C57BL)	8	17	16 hr	Embryonic gut
9 (C57BL)	8	22	16 hr	Hindgut of some embryos
10 (C57BL)	9	9	4 hr	
11 (c57BL)	10	12	8 hr	No embryonic uptake
11 (C57BL)	10	12	8 days	No embryonic uptake
16 (C57BL)	15	12	4 hr	No embryonic uptake High in chorioallantoic placenta
35 days prior to mating			53 days	No uptake in embryo or placental structures

ovaries, oviducts and cervix were removed. The uterus was then placed in a horizontal plane on a microtome stage, embedded in an aqueous gel of carboxymethyl cellulose (CMC) and frozen by immersion of the stage in a mixture of solid carbon dioxide and hexane (-78°C). To estimate the relative uptake in the uterus and the embryos in relation to the other maternal organs, pieces of the liver and kidney (known to accumulate cadmium) were removed and embedded in the CMC together with the uterus. The hamster killed after 4 days (injected on the 8th day) was embedded intact in the CMC and frozen as described above. In the mouse

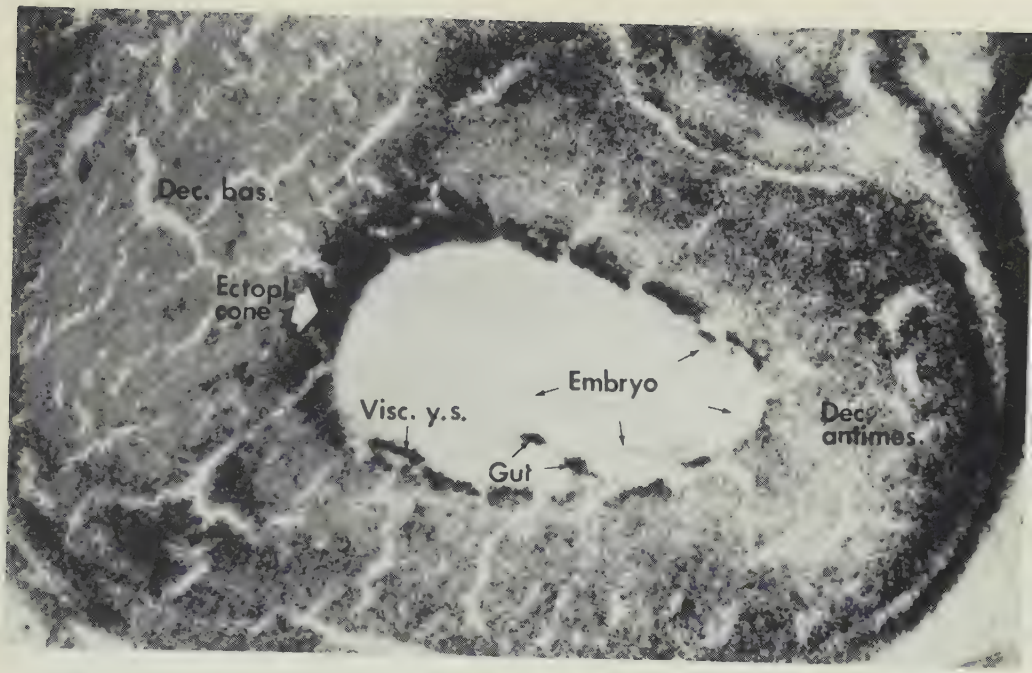
injected on the 11th day and killed 8 hr later and the one injected on the 16th day and killed 4 hr later, only half of each uterus was removed and embedded and the mother's abdominal wall was then sutured. These mothers were subsequently embedded intact. According to the method of Ullberg (1954, 1958), sagittal 10 µm and 20 µm sections, attached to tape (No. 800, Minnesota Mining and Manufacturing Co.), were cut in a microtome at -15°C through the frozen animals or uteri (in a horizontal plane through as many embryos as possible) and were dried at the same temperature. The dried sections were then transferred to room temperature. Exposure was achieved by apposition against roentgen film (Industrex C, Kodak). The exposure time ranged from 10 to 190 days. Selected sections were stained with hematoxylin and eosin and mounted on glass slides in Euparal (Flatters & Garnett Ltd, Manchester, England) under a coverslip.

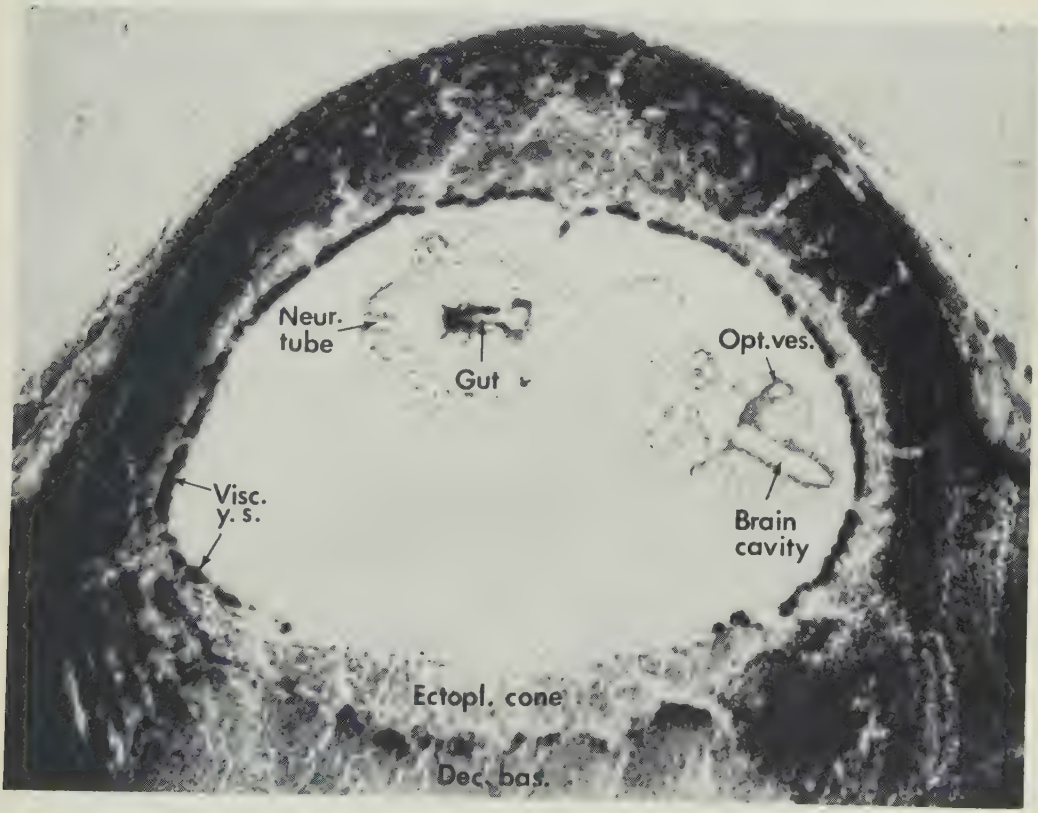
RESULTS

The overall distribution pattern of cadmium in the hamsters and mice was essentially the same, and was in accordance with that described by Berlin & Ullberg (1963 b). The most prominent accumulation was seen in the liver and the renal cortex. The findings concerning the embryonic (fetal) uptake are summarized in table I:1.

Fig. I:1 (on the opposite page, upper). Photomicrograph of a section with the superimposed autoradiogram (black grains representing radioactivity). It is a part of a uterus of a hamster 4 hr after the injection of $^{109}CdCl_2$ on the 8th day of gestation (7 days 14 hours), showing one embryo. Note the high uptake in the primitive gut, the visceral yolk sac and in the ectoplacental cone. Unstained section (× 20).

Fig. I:2 (on the opposite page, lower). Photomicrograph of a section with the superimposed autoradiogram (black grains). It is a part of a hamster uterus 24 hr after the injection of $^{109}CdCl_2$ on the 8th day of gestation (7 days 14 hours), showing an embryo. Note the retention in the gut wall of the embryo and in the visceral yolk sac. Unstained section (× 22).


Dec. bas.
Ectopl. cone
Embryo
Visc. y.s.
Gut
Dec. antimes.

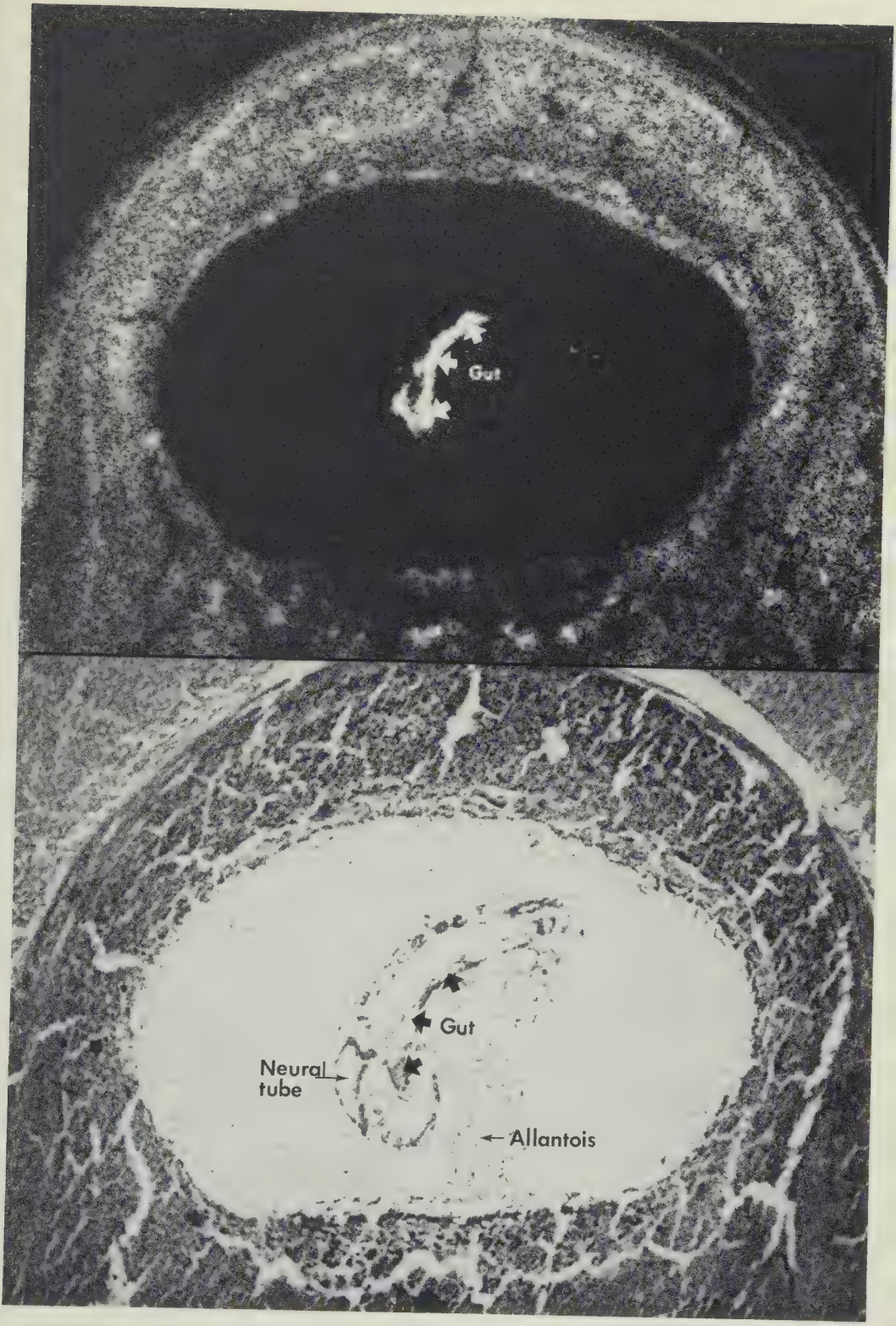

Neur. tube
Gut
Opt.ves.
Visc. y. s.
Brain cavity
Ectopl. cone
Dec. bas.

Embryonic accumulation. There was a marked variation with time in the embryonic uptake of cadmium. In the embryos of the animals injected on the 8th or on the first half of the 9th days of gestation (up to 8 days 17 hours after mating), a considerable accumulation was seen at all survival intervals in a structure which seemed to represent the primitive gut (figs I:1-3). Radioactivity was seen in the alimentary tract still 4 days after the injection. No radioactivity was seen in any other embryonic structure. The injection at gestational ages later than 8 days 17 hours did not result in any embryonic uptake (fig. I:4).

Placental accumulation. Four hr after the injection on the 8th day of gestation, a heavy accumulation of ^{109}Cd was observed in the visceral yolk sac and in the ectoplacental cone (fig. I:1). The antimesometrial decidua had a moderate concentration. A high concentration of ^{109}Cd was still present in the visceral yolk sac 24 hr after the injection (fig. I:2). In the developing chorioallantoic placenta, the concentration was low, but at its junction with the decidua basalis activity was seen in the cells of the ectoplacental cone (fig. I:2). Four days after the injection (the mouse killed at day 12 of gestation) the ^{109}Cd concentration was low in all the placental structures. After the injection of cadmium on the 9th day of gestation, a high concentration was noted in the visceral yolk sac and the ectoplacental cone and a moderate concentration in the developing chorioallantoic placenta (fig. I:4).

Ovarian accumulation. There was a heavy accumulation of cadmium in the corpora lutea and in the thecal cells of the follicles, and a lighter accumulation in the granulosa cells and the interstitium the first 24 hr after injection (fig. I:5). Four days after the injection (12th day of gestation), there was still a high concentration in the corpora lutea, while it was somewhat lower in the follicular walls. In the interstitium, areas (possibly atretic follicles) with a high cadmium content were seen.

Fig. I:3 (on the opposite page). Autoradiogram (upper) and the corresponding section (lower) of a hamster uterus, showing an embryo, 24 hr after the injection of $^{109}CdCl_2$ on the 8th day of gestation (7 days 20 hours). Note that there is an accumulation in the embryonic gut (white areas) which is considerably higher than in the surrounding uterus and placental structures. No activity can be seen in the other structures of the embryo. Hematoxylin-eosin (× 24).


Gut
Gut
Neural tube
Allantois

Embryonic accumulation. In the series of mice injected on the 8th and 9th days of gestation (up to 8 days 17 hours and in some embryos 8 days 22 hours after mating), embryonic uptake was seen up to 24 hr later. Radioactivity was still noted in some embryos 3 days after injection. At 5, 7, 9 and 11 days after injection, no fetal cadmium was seen. The localization was the same as that described for the golden hamster (fig. I:6). One embryo out of eight of the mouse injected at day 10 (9 days 9 hours after mating) showed a gut accumulation. In the two mice injected at the 11th day of gestation that were allowed to survive for 8 hr and 8 days, respectively, no embryonic uptake was observed. The same was true for the mouse injected on the 16th day of gestation that was killed 4 hr later (fig. I:7) and the mouse injected 35 days before mating and killed on the 19th day of gestation (53 days survival time) (fig. I:8).

Placental accumulation. There was a heavy accumulation of ^{109}Cd in the decidua in the antimesometrial part of the uterus of the mice injected on the 8th and 9th days of pregnancy (fig. I:6). This was high up to 24 hr after injection and then gradually diminished so that in the animals killed at a late stage of gestation (after injection in early gestation), only spots of radioactivity were noted in this part of the uterus. There was also some uptake in the visceral yolk sac. Injection on the 10th and the 11th days of gestation (4 and 8 hr survival time respectively) resulted in an antimesometrial accumulation and this also occurred in the circumference of the fetus, apparently in the omphalopleure and in the decidua capsularis adjacent to it. The placental cone and the visceral yolk sac also showed a considerable accumulation. Eight days later, spots of radioactivity were seen in the decidua basalis, sometimes surrounding the maternal blood vessels, and in the uterine wall. In the mouse injected at the 16th day of gestation and killed 4 hr later, there was a heavy accumulation of ^{109}Cd in the placental labyrinth (fig. I:7) which was comparable to the concentration in the maternal liver. In the decidua basalis, spots of

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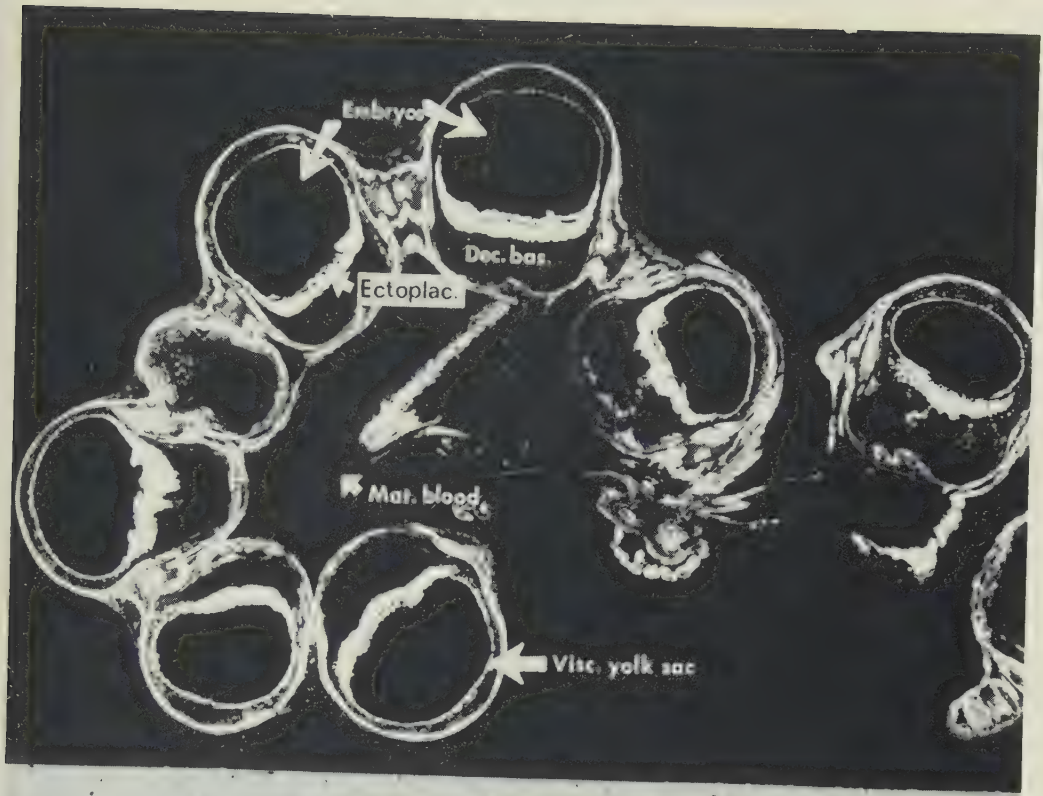
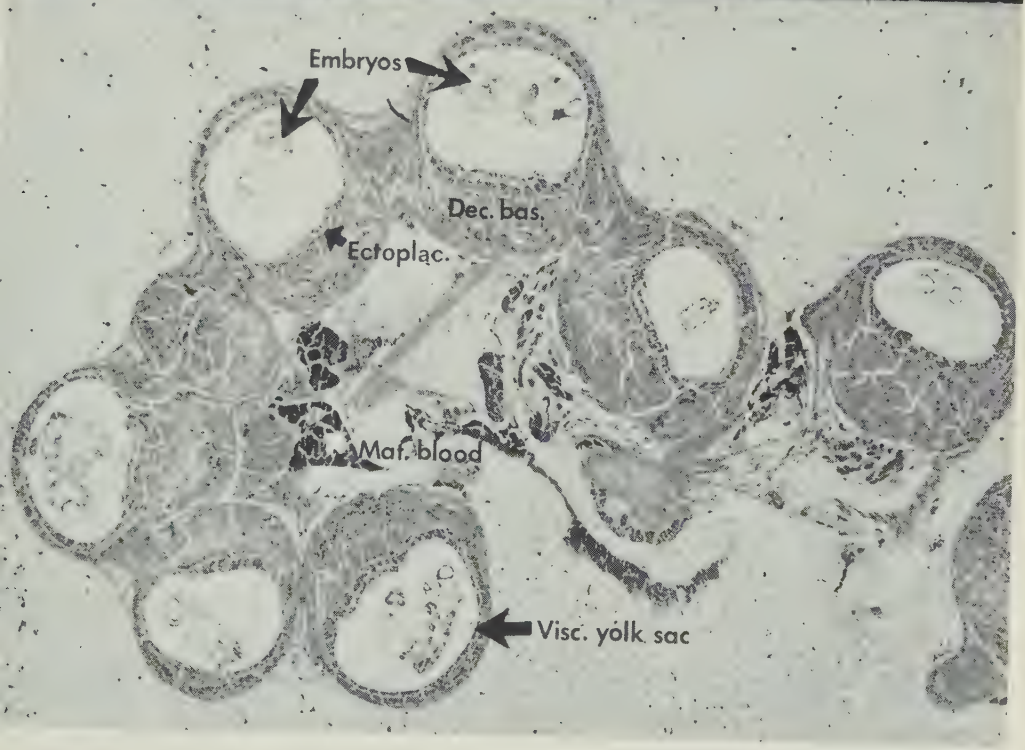
Fig. I:4 (on the opposite page). Autoradiogram (upper) and the corresponding section (lower) showing the whole uterus of a hamster 4 hr after the injection of $^{109}CdCl_2$ on the 9th day (8 days 17 hours). The embryos are cut at different levels. No radioactivity can be seen in the embryos. There is however a heavy accumulation in the ectoplacental cone and the visceral yolk sac. Hematoxylin-eosin (× 4).


Embryos
Dec. bas.
Ectoplac.
Mat. blood
Visc. yolk sac


Embryos
Dec. bas.
Ectoplac.
Mat. blood
Visc. yolk sac

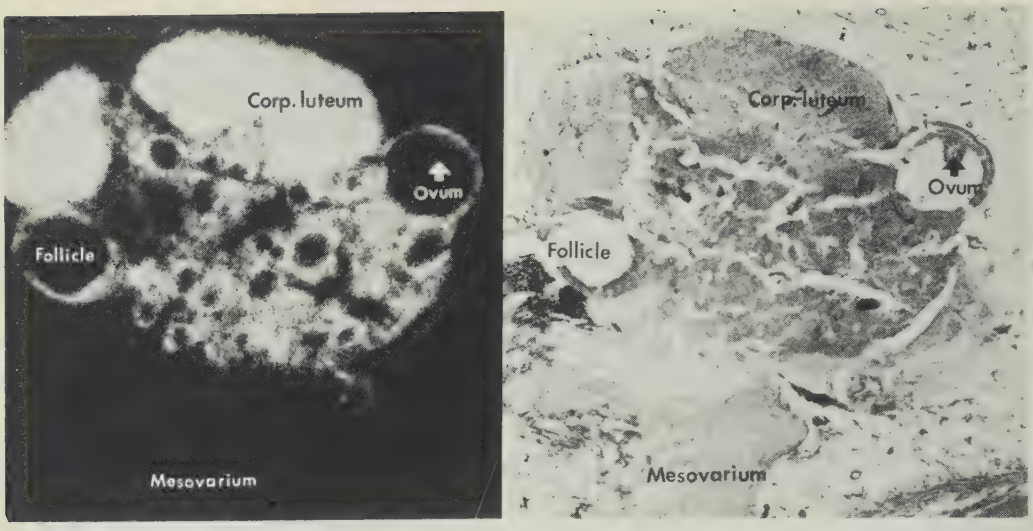
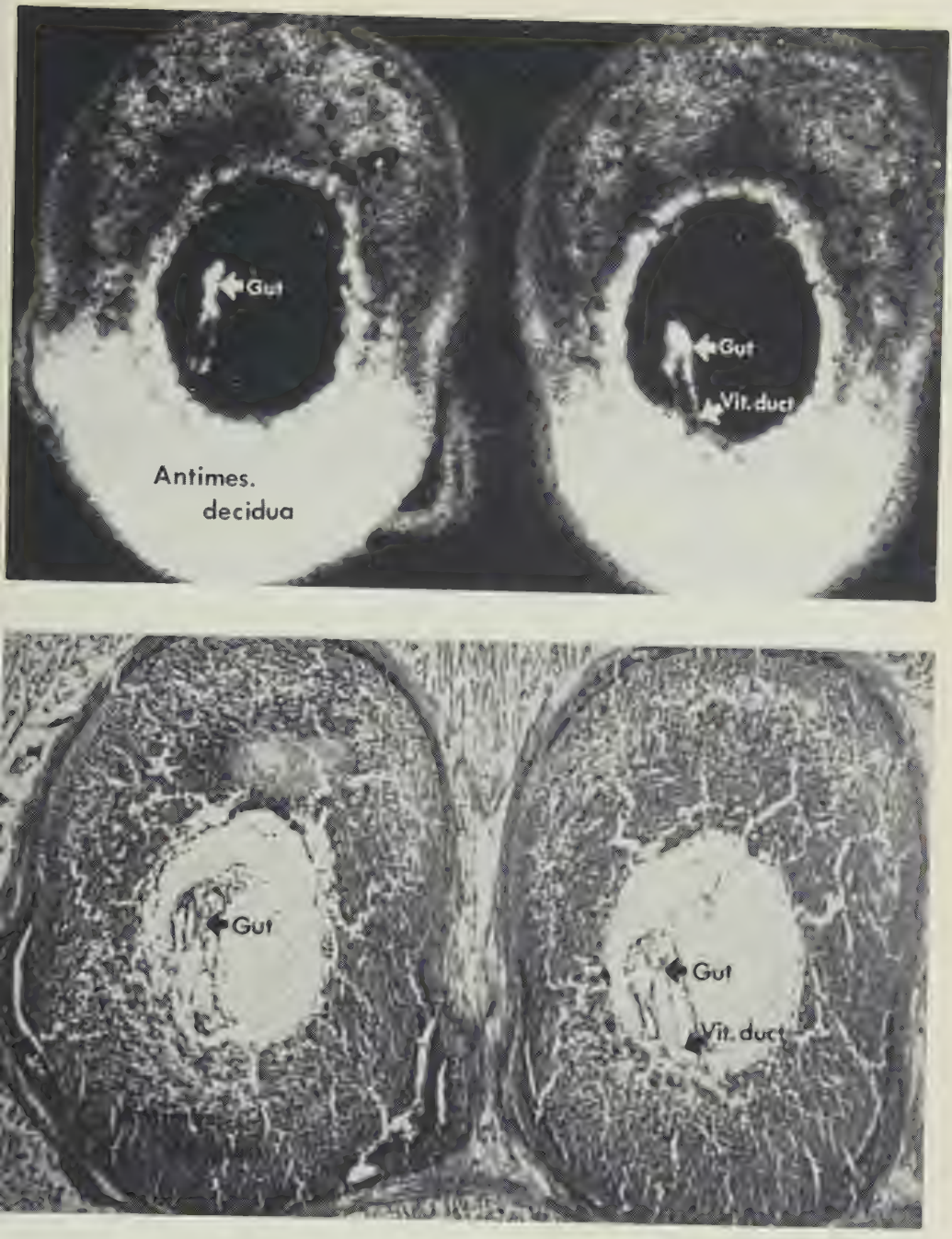


Fig. I:5. Autoradiogram (left) and the corresponding section (right) showing a hamster ovary 24 hr after the injection of $^{109}CdCl_2$ on the 8th day. Note the high uptake in the corpora lutea and the follicular walls. It can be seen that the cumulus oophorous and the ovum are almost devoid of radioactivity. Hematoxylin-eosin (× 15).

cadmium uptake were seen as in the mouse killed on the 19th day of gestation. In the mouse injected 35 days before mating (fig. I:8) the placental and uterine concentrations were very low. In contrast to the decidua, however, the placental labyrinth showed a weak uptake.

Ovarian accumulation. As in the hamster, the injection of cadmium into the mouse resulted in an ovarian accumulation, although it was not equally marked. In all mice, at short survival intervals (up to 24 hr), there was moderate concentration in the corpora lutea and in the follicular walls. Three days after injection, there was still some radioactivity in these structures. Later, areas of high concentration (possibly atretic follicles) were seen in the interstitium. In the mouse which was killed after 53 days (fig. I:8), a high concentration was still seen in the interstitium.

Pituitary. In the hamster and the mouse, there were marked accumulations of cadmium in the pituitary (figs I:7 and 8).



ig. I:6. Autoradiogram (upper) and the corresponding section (lower) howing a part of a mouse uterus with two embryos. It is 8 hr after he injection of $^{109}CdCl_2$ on the 9th day of gestation (8 days 13 ours). There is a high accumulation in the embryonic gut, and con- inuous with that in the vitelline duct and visceral yolk sac endo- erm. Hematoxylin-eosin (× 21).

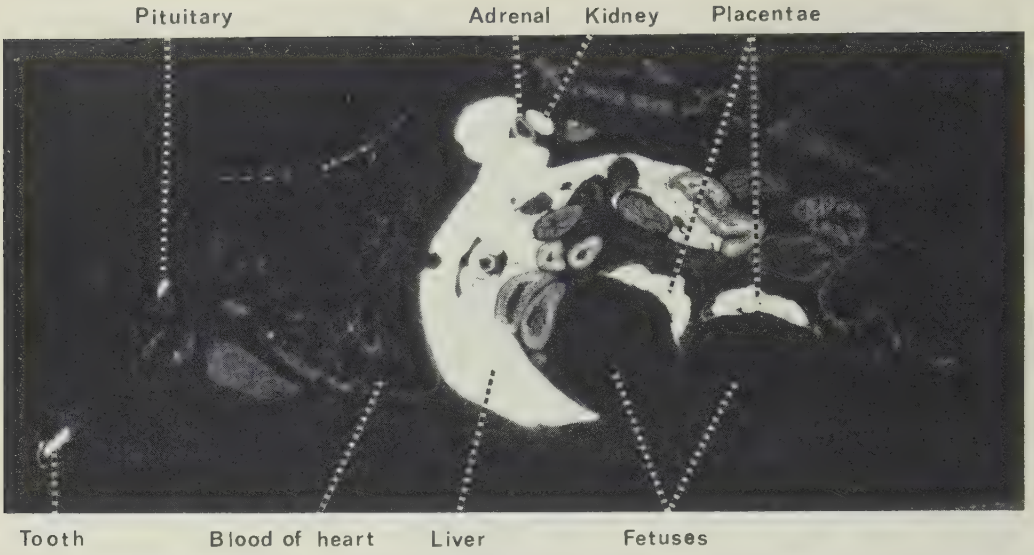


Fig. I:7. Whole body autoradiogram of a mouse 4 hr after the injection of $^{109}CdCl_2$ on the 16th day of gestation. Note the absence of radioactivity in the fetuses but the heavy accumulation in the chorioallantoic placentae (comparable with the liver and the kidney). Also the pituitary and the adrenal cortex show an uptake.

DISCUSSION

Embryo

The present investigation showed that cadmium passed to the embryo and strongly accumulated in the primitive gut only when injected before a certain stage of gestation, after which there was essentially no uptake.

It has been shown in the rabbit that Evans blue attached to maternal serum proteins can be detected in the yolk sac cavity by the 7th to 8th day of gestation (Brambell & Hemmings 1949). The subcellular structures of Reichert's membrane and the parietal yolk sac endoderm in the rat (days 7 to 10) show that serum proteins may pass through these membranes into the yolk sac cavity (Merker & Villegas 1970). This is probably true also for the golden hamster and the mouse due to the similarities in the formation of these embryonic membranes. In mice, a short time after the administration of a single

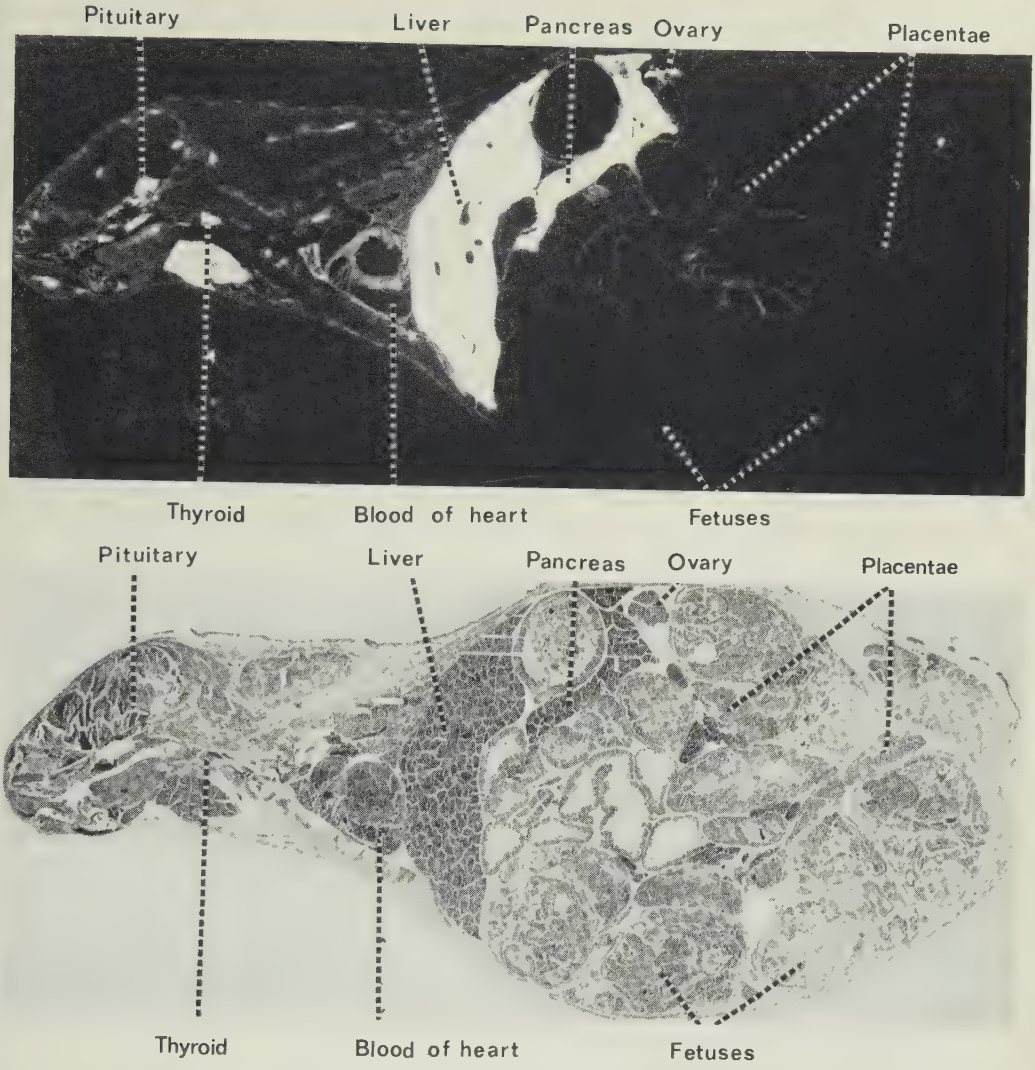


Fig. I:8. Whole body autoradiogram (upper) and the corresponding section (lower) of a mouse on the 19th day of gestation. It was injected with $^{109}CdCl_2$ 35 days prior to conception. Note the very weak uptake in the placentae and the absence of cadmium in the fetuses. There is a considerable accumulation in the pituitary, the thyroid and in the ovarian interstitium.

dose, cadmium was found in the serum fractions corresponding to the size of albumin or the larger proteins (Nordberg 1972). The ion may be transferred into the yolk sac cavity bound to such proteins. The high uptake found in the visceral yolk sac endoderm also favours such a transfer; cadmium (probably bound to proteins) had been absorbed by this endodermal tissue from the yolk sac cavity.

In the early postimplantation stages there is free communication between the primitive gut and the yolk sac cavity, which makes the passage of yolk sac material into the gut lumen possible, after which it can be absorbed by the gut endoderm. During days 8 and 9 of gestation, both in the mouse and the hamster, the considerable structural development of the embryo results in the closure of this communication. This has been shown to be completed 9 to 9 1/4 days after conception in the hamster (Boyer 1953). It is probable that this communication had functionally disappeared at the time of injection on the 9th day (at 8 days 17 hours or at least 21 hours after mating) in the hamster in this investigation. In the mouse, this so-called 'vitelline duct' is closed 9 1/4 days after conception (Rugh 1968). The injection of ^{109}Cd at this stage resulted in gut accumulation in only one embryo out of eight. Agreement thus seems to exist between the degree of communication through the vitelline duct and the observed uptake in the gut wall.

It is clearly seen in the autoradiograms and it is notable that - even at long survival intervals after the injection - there was a retention in the gut wall but no radioactivity was observed in any other organ. From different parts of the primitive gut, many essential organs are formed by the interaction of the surrounding mesenchyme: the Eustachian tubes, palatine tonsils, thyroid and parathyroid glands, thymus, lungs, liver and pancreas. Endodermal damage by the accumulation of cadmium may hinder the normal formation and differentiation of these organs and the digestive tract, but the possible teratogenic effects of cadmium on these organs require further investigations. The profound damage to the facial anatomy of hamsters produced by cadmium (Gale & Ferm 1973) when given on the 8th day of gestation may be the result of a failure in the stomodeum and the foregut to interact as the cadmium is accumulated in the gut. The cadmium damage to other structures of mesodermal origin, e.g. bone structures (Gale & Ferm 1973), is more difficult to explain on the basis of an endodermal accumulation, even if one always has to reckon with different tissue interactions.

It seems possible that long-term retention in the gut wall may affect intestinal function with secondary damage to all the organism. The role of the intestine in early intrauterine life is not well known. In the human fetus the gut is already opened to the stomodeum by the end of the third week (Langman 1969) and in the mouse by day 9 (Rugh 1968). At 6 to 7 weeks muscles and nerves are seen in the gut wall of human embryos (Becker et al. 1940). Fetal swallowing has been observed at 12 weeks of age (Mori 1956) and at mid-pregnancy (Abramovich 1970). Intestinal lysosomal activity in 10-week-old embryos is the same as after birth (Antonowicz et al. 1974). As the gut endoderm is derived from the visceral yolk sac endoderm, it seems probable that these two structures develop the same role for the absorption of nutrients to the fetus (the yolk sac mainly in rodents) from the amniotic, and the uterine cavities, respectively.

Gale & Ferm (1973) have carefully shown the changes in the teratogenic effects of cadmium with time of gestation. At day 8 it produced resorptions and severe facial, brain, eye and limb malformations. When administered in the afternoon on the 9th day no resorptions and only minor reductions of the ossification centers occurred. This is the time when cadmium passage to the embryo stopped according to the present study. It may indicate that the early severe damage is due to a direct effect on the embryo. The present finding is of fundamental interest because the same transport route into the embryo may be utilized by other substances of teratogenic importance, and also by those which are blocked by the placenta during later gestational stages. In man a communication persists between the yolk sac cavity and the primitive gut up to about 1 month (Langman 1969). As the yolk sac is large (and probably functionally important) during the first weeks of pregnancy, these experimental findings may also have some relevance in man.

Decidua and placental structures

The variation in sensitivity to teratogens in general is considered to be a function of the degree of organ development (see e.g. Degenhardt & Kleinebrecht 1971). This is probably true also for cadmium and therefore extraembryonic mechanisms may also be considered as involved in the embryonic damage. Many cations, among them cadmium, bind to the thiol groups on enzymes which are essential for the enzyme activity (Hewitt & Nicholas 1963). Cadmium is known to inhibit many enzymes (Kench & Gubb 1970; Unger & Clausen 1973). Progesterone

induces a growth of the endometrium and a change in its enzyme content preparatory to implantation (Hafez 1964). Histochemical methods have shown the presence of proteolytic enzymes in the antimesometrial decidua on the 8th day in the mouse (Bergström 1972), the localization of which was in accordance with that of cadmium in the mouse at the same stage in the present investigation. A possible enzyme affinity for cadmium may influence the maternal-embryonic relationship, and result in an inhibited implantation, resorptions or malformations.

The visceral yolk sac plays an essential role in the nutrition of the early embryo and the well-known teratogen, trypan blue, is believed to act by inhibiting this nutrition (see e.g. Lloyd & Beck 1969). The accumulated cadmium may also disturb vital enzyme systems in this structure. In the chorioallantoic placenta during late gestation in the mouse, there was a heavy accumulation of cadmium which was as high as that in the maternal liver and kidney. This uptake may be related to the numerous placental enzymes (Hafez 1964). It probably explains the extensive placental necrosis with the subsequent fetal death caused by cadmium, which has been observed in the structurally related rat placenta in late gestation (Pařizek 1964). Mice injected 35 days before conception had very low concentrations of cadmium in the late stage placenta, indicating an insignificant release of this metal from other organs. This probably means that the risk of fetal damage by cadmium accumulated in the mother before gestation is low. The finding is in accordance with that of Webb (1972).

Cadmium is accumulated in the steroid-producing organs, viz. the interstitial parts of the testes (Berlin & Ullberg 1963 b) and the follicles and corpora lutea (see below). The decidual accumulation seen in mice at late gestation may be compared with the pattern of 3β-hydroxysteroid dehydrogenase activity described earlier in rats and mice (Deane et al. 1962; Botte et al. 1966). Appelgren (1967) reported an intense accumulation of pregnenolone in the mouse decidua, especially in the cells surrounding the maternal blood lacunae. Botte et al. (1966) found 17α- and 17β-hydroxysteroid dehydrogenase activity in the visceral yolk sac endoderm where cadmium also accumulated.

Ovary

It is known that ovariectomy causes fetal resorptions (before days 15 to 16) or abortion (later) in the mouse (Harris 1927). Removal of the corpora lutea also results in termination of the pregnancy (Parkes 1928) while progesterone will maintain pregnancy in castrated

rabbits (Wu & Allen 1959).

The accumulation of cadmium in the corpora lutea and follicles, especially in the thecal cells, resembles that of pregnenolone and cholesterol which also accumulated in the interstitium (Appelgren 1967). Appelgren found 3β-hydroxysteroid dehydrogenase activity especially in the corpora lutea and in the follicular thecal cells. These similarities may indicate that cadmium accumulation is dependent on an affinity for the steroid hormone forming enzyme systems. Cadmium, in addition, decreases the activity of cytochrome P-450 (Unger & Clausen 1973) which is involved in steroid biosynthesis. Cadmium intoxication leads to reversible damage to the ovary (bleeding and follicular atresia) (Kar et al. 1959; Kaul & Ramaswami 1970) and an irreversible injury of the testes (Pařizek 1957). This has been suggested as being secondary to injuries of the vessels (Chiquoine 1964; Gunn et al. 1966; Johnson 1969), but it is also known that cadmium influences the testicular androgen production *in vitro* in the trout (Sangalang & O'Halloran 1973).

Changes in the gonadotrophic cells of the adenohypophysis have been described as a result of 'cadmium castration' (Allanson & Deanesly 1962; Girod 1964; David & Ramaswami 1971). It is probable, however, that the changes are partly a direct effect due to the accumulation of cadmium in these cells.

This study does not conclusively determine the site of teratogenic action of cadmium. It illustrates many sites of accumulation, the significance of which for the developing fetus may be summarized as follows:

1. The heavy accumulation in the embryonic gut wall in early gestation makes probable a direct effect on the embryo.

2. The decidual and placental uptake indicates that the maternal-embryonic relationship, and therefore the embryonic nutrition, may be disturbed.

3. The ovarian accumulation may disturb steroid synthesis leading to an interruption of the pregnancy.

CHAPTER II

TRYPAN BLUE: ACCUMULATION IN THE GUT OF RODENT EMBRYOS DURING TERATOGENIC PHASE

Although not of importance for human fetal development the bisazo dye trypan blue has become one of the most extensively used experimental teratogens and a model substance for causing teratogenic damage by extraembryonic mechanisms. Gillman et al. (1948) who showed its teratogenic action in the rat found that the dye did not pass the placenta and suggested that it caused some maternal metabolic defect which secondarily damaged the embryo. Since then several authors have observed the deposition of the trypan blue in the visceral yolk sac endoderm (Wilson et al. 1959; Barber & Geer 1964; Beck et al. 1967; Lloyd & Beck 1969). Furthermore, Beck et al. (1967) showed that the dye inhibited the lysosomatic enzymes of the visceral yolk sac endoderm and suggested a mechanism based on the inhibition of embryotrophic digestion in the visceral yolk sac and the possible effect this could have on embryonic nutrition.

Ferm (1956) found trypan blue in the blastocoelic fluid in the rabbit. Traces of the dye have also been found in the embryo (Barber & Geer 1964; Davis & Gunberg 1968; Greenhouse et al. 1969). However, Wilson et al. (1963) using ^{14}C-labelled trypan blue did not find radioactivity in the embryo. The positive findings of trypan blue in the embryo therefore have not received much attention.

In chapter I the distribution of ^{109}Cd in the uteri and embryos of mice and golden hamsters was studied by means of autoradiography. The cadmium passed to the embryo and accumulated in the wall of the primitive gut - where it was retained. This occurred during a limited stage of development when there is a free communication between the yolk sac cavity and the embryonic gut. This stage largely coincides with cadmium's period of teratogenic sensitivity according to studies made in the golden hamster published by Ferm and co-workers (Ferm 1971; Gale & Ferm 1973). Later in gestation the cadmium did not pass to the embryo (fetus) in detectable amounts.

This finding suggested that a comparative study of other substances might be of interest in regard to their possible passage to the embryo when the above mentioned communication is still open. It

seemed pertinent to choose trypan blue as it exerts its teratogenic action only in the early organogenetic period - in analogy with cadmium. The mouse, hamster and rat were studied with a histological technique using cryostat sections taken through the whole uteri after the injection of teratogenic doses of trypan blue. To avoid the loss or redistribution of the dye, the sections were freeze-dried and viewed directly in the microscope. Radioactive cadmium was also injected into two of the mice in order to compare the distribution of the trypan blue and the cadmium in the same animal.

MATERIALS AND METHODS

Trypan blue (puriss.) was obtained from Chroma-Gesellschaft, Schmid & Co., Stuttgart, West Germany. $^{109}CdCl_2$ (spec. act. 166 μCi/μg) was obtained from The Radiochemical Centre, Amersham, England. Animals used were the following: mice of the C57BL strain, 3 months old; rats of the Sprague-Dawley strain about 6 months old; albino golden hamsters, 3 months old. The animals' environmental conditions and the mating procedure for the mice and the hamsters were described in chapter I. The rats were mated overnight, and a vaginal smear was examined in the microscope for the presence of sperms.

The trypan blue was dissolved in distilled water. The dose given to all animals was 50 mg/kg body weight. The mice were injected intravenously (0.2 ml), the rats subcutaneously or intravenously (0.5 ml) and the hamsters intravenously (0.25 ml) under light ether anesthesia. Two mice were injected intravenously with $^{109}CdCl_2$ immediately before the trypan blue injection. They received 5 μCi or 0.03 μg dissolved in 50 μl 0.1 M-HCl. The injection schedule is seen in table II:1. The gestational age was determined as described in chapter I for the mice and hamsters by the assumption that fertilization occurred on an average at 24.00 hours in the mice and the rats and at 20.00 hours in the hamsters.

The animals were killed and ovariohysterectomized. The uteri and kidneys were mounted and sections of 20, 40 and 60 μm were cut, attached onto tape as described. The dried sections were transferred to room temperature in an air tight box to prevent condensation of

TABLE II:1. Injection regimen of trypan blue into pregnant rats, mice and a golden hamster and the subsequent uptake of the dye in the embryonic gut.

Day of gestation on which ^{109}Cd injected	*Interval between conception and injection*		*Interval between injection and autopsy*	*Route of administration*	*Embryonic gut accumulation*
	Days	*Hours*	*Hr*		
Rat					
9	8	17	16	i.v.	(+)
9	8	22	16	i.v.	(+)
10	9	9	4	i.v.	(+)
10	9	9	24	s.c.	+
10	9	13	4	i.v.	++
10	9	16	16	s.c.	+
10	9	20	4	i.v.	++
11	10	9	4	i.v.	++
11	10	9	4	i.v.	++
11	10	13	4	i.v.	++
12	11	8	4	i.v.	-
16	15	8	4	i.v.	-
Mouse					
8	7	17	4	i.v.	(+)
8	7	17	24	i.v.	+
8	7	17	48	i.v.	-
8	7	21	12	i.v.	+
8	7	21	12	i.v.	+
8	7	21	24	i.v.	+
9	8	7	4	i.v.	++
9	8	7	4	i.v.	++
9	8	7	8	i.v.	+
9	8	9	4	i.v.	+
9	8	10*	1	i.v.	(+)
9	8	10*	4	i.v.	++
9	8	20	12	i.v.	(+)?
10	9	9	4	i.v.	-
Hamster					
8	7	15	4	i.v.	+

*injected with $^{109}CdCl_2$ prior to trypan blue administration

humidity onto the tissues. Thereafter the sections of the mice injected with $^{109}CdCl_2$ were submitted to autoradiography as described in chapter I. All sections were then viewed unstained in a microscope at a low magnification. Microphotographs were taken in a Zeiss photomicroscope using an orthochromatic film (Kodalith ortho film, type 3, Kodak). The specimens were illuminated both with transmitted light

and with combined transmitted and reflected light. The latter procedure considerably increased the light absorbing capacity of the tissues intravitally stained with trypan blue.

To test the effects of different fixatives, selected sections were immersed in 4 per cent formaldehyde in a phosphate buffer (pH 7,4) or 5 per cent trichloroacetic acid for 2 minutes at 4°C. The fixed sections were thereafter rinsed in water for 10 minutes, dried at room temperature and subsequently examined in the microscope.

RESULTS

General observations

In all dams a heavy accumulation of the dye was seen in the connective tissues of the skin and muscles. The blue colour was quenched by the blood of the parenchymatous organs, but these had a darker colour than normal.

In the thin freeze-sections through the uteri and the kidneys these tissues appeared pale blue to the naked eye. In the microscope an accumulation of the blue dye could be seen in certain tubules of the renal cortex, probably the proximal tubules. In the ovaries the follicular fluid was coloured blue. The uterine wall and the decidua were coloured in a characteristic pattern. In the decidua the antimesometrial region and that close to the embryo were relatively free of the dye.

Embryo and visceral yolk sac endoderm

The main findings of embryonal uptake are summarized in table II:1.

(a) *The period before the embryonal gut formation* (in this study up to 9 days 9 hours in the rat and 7 days 17 hours in the mouse).

A constant finding in both the mouse and the rat was that the ectodermal and mesodermal layers of the embryos were free of the trypan blue at all stages (fig. II:1). The proximal endoderm of the extraembryonic part and what seems to be the foregut and hindgut areas showed a heavy accumulation of the dye, while it was lower in the part surrounding the rest of the embryo. At the most

antimesometrial point, the dye was accumulated in a dilated part of the yolk sac cavity. An accumulation was seen in the yolk sac cavity not only at this stage but also later.

(b) *The period from the beginning of gut formation to the closure of the vitelline duct* (in this study from 9 days 9 hours to 10 days 13 hours in the rat and from 7 days 21 hours to 8 days 20 hours in the mouse).

At this period the trypan blue was clearly visible in the gut lumen and wall (figs II:2-4). This was most obvious a short time after the injection of the dye (4 hr) but was also evident at 16 and 24 hr. At 48 hr, however, no retention could be observed.

The relatively thick sections, which were used in order to increase the possibilities of detecting the dye, were not always of a quality that favoured the identification of the structures of the embryo. However, the dye within the embryo was always restricted to a limited area, the position of which made it probable that it represented the gut. In the experiment where both trypan blue and $^{109}CdCl_2$ were injected, the dye was observed in the same structures which showed the radioactivity (fig. II:5). This finding confirms the assumption that the dye was localized in the gut. This confirmation does not exclude the possibility that the dye had passed the gut endoderm and localized in small concentrations in other tissues, because detection was rather limited.

There is no good method to quantify the amount of dye in the embryonic gut. Since the visceral yolk sac endoderm surrounding the embryo has been extensively studied this may be a good reference. The concentration of the dye in the gut wall usually did not reach that of the visceral yolk sac endoderm. However the concentration was almost as high at certain short survival times (fig. II:3). Sometimes the dye had a granular appearance, but usually the gut wall was coloured evenly blue without the spotty accumulation found in the visceral yolk sac endoderm. Often the concentration in the embryonic gut could be compared to that of the renal cortical tubules showing a medium accumulation.

(c) *The period after vitelline duct closure* (from 11 days 8 hours in the rat and 9 days 9 hours in the mouse).

In the embryos of animals injected at 11 days 8 hours (rat) and 9 days 9 hours (mouse) no dye was found in the gut (possibly a

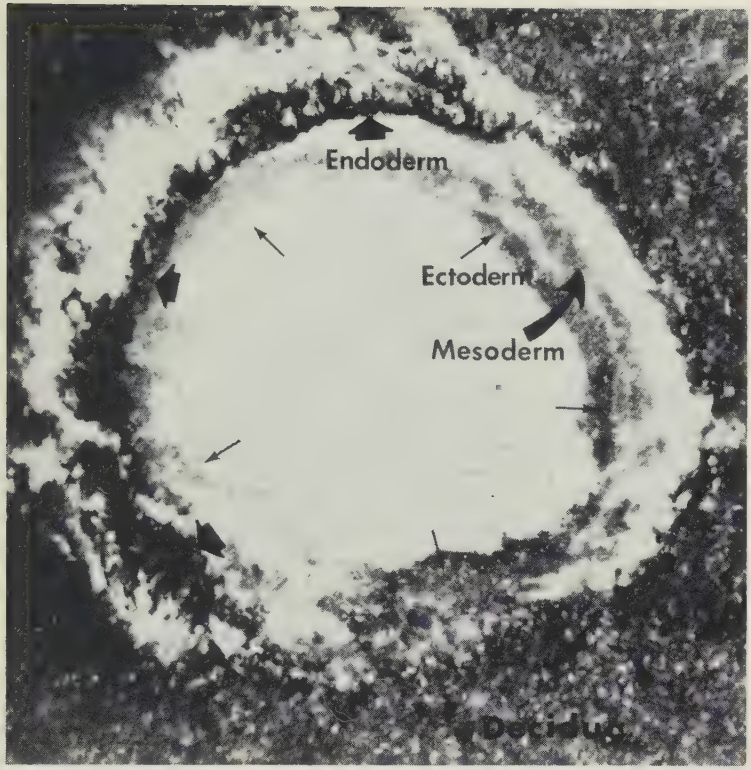


Fig. II:1 (upper). Photomicrograph of an unstained freeze-section through a part of a rat uterus 4 hr after the injection of trypan blue in a rat at 9 days 9 hours of gestation. Note the heavy dark colour in the endodermal layer, but the absence of colour in the ecto- and mesodermal tissues (× 118).

Fig. II:2 (left). Photomicrograph of an unstained freeze-section through the caudal part of a rat embryo 4 hr after the injection of trypan blue in the dam at 10 days 13 hours of gestation. There is a high accumulation of the dye in the gut but no colour can be detected in the other tissues (× 118).

small amount was seen in one mouse embryo). At these stages the vitelline duct is closed or has at least disappeared as a functional communication between the yolk sac cavity and the embryonic gut lumen. In addition, no dye was detected in the late stage fetuses of the rat (day 16).

Although seven hamsters were injected, only one was considered accountable in this study. In this one the dye was seen in the embryonic gut similarly to what was found in the rats and mice. In the others the degree of embryonic resorption was too high.

Effects of fixatives

When immersed in a formaldehyde solution, the section lost most of the blue colour. This was true for the embryonic gut, the yolk sac epithelium and the renal tubules as well as the other structures. The fixation in trichloroacetic acid, however, preserved most of the dye in the structures and fluids where it was originally bound.

DISCUSSION

The results of the present investigation of trypan blue and earlier findings concerning cadmium (chapter I) show that these two teratogenic agents pass into the fetus in a similar way. Both trypan blue (Rawson 1943) and cadmium (Nordberg 1972) are to a great extent bound to the serum proteins, which probably pass the Reichert's membrane and the parietal yolk sac endoderm into the yolk sac cavity (Brambell & Hemmings 1949; Merker & Villegas 1970). The trypan blue was also seen in the yolk sac fluid in this investigation.

In this way the embryonic endoderm - i.e. the future intestinal epithelium - is exposed to these teratogens up to the time when the

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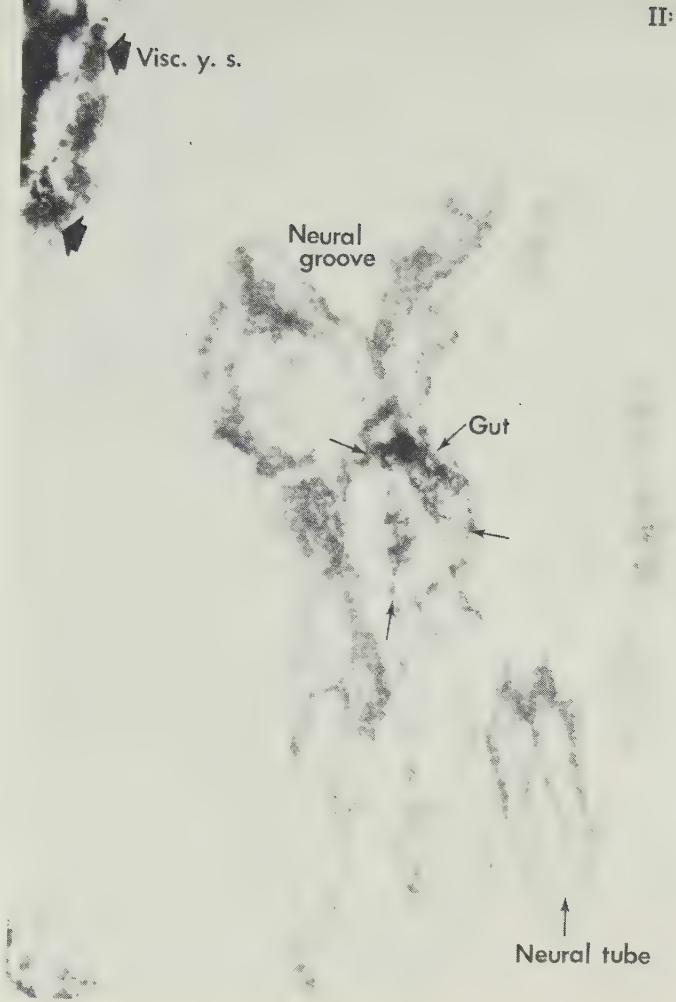
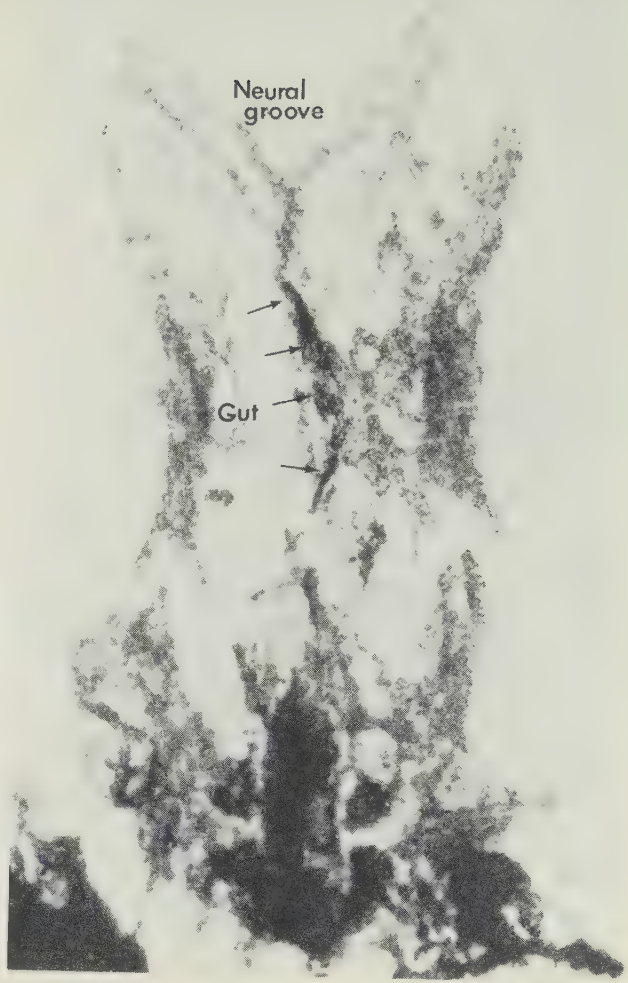
Figs II:3 and 4 (on the opposite page). Photomicrographs of unstained freeze-sections through a mouse uterus 4 hr after the injection of trypan blue at 8 days 7 hours of gestation, showing two different embryos and parts of the surroundings. In both the embryos an accumulation of the dye can be seen in the gut, which is as high as that in the visceral yolk sac endoderm (fig. II:3, upper left). The dark areas in fig. II:4 (bottom) correspond most to blood (× 112).

II:3



II:4



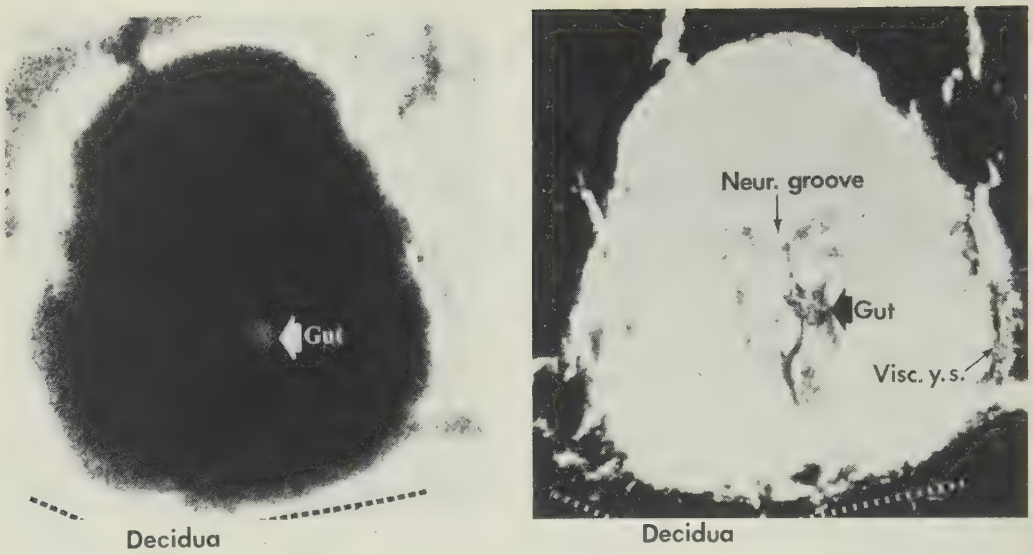


Fig. II:5. Autoradiogram (left) and the corresponding unstained freeze-section (right) of a mouse uterus 4 hr after the simultaneous injection of $^{109}CdCl_2$ and trypan blue at 8 days 10 hours of gestation. An accumulation of radioactive cadmium (white spot marked by an arrow) in the autoradiogram corresponds to the gut of the embryo in the section, where trypan blue was accumulated (× 32).

vitelline duct communication functionally disappears. As was earlier shown for cadmium, the trypan blue accumulated in the wall of the embryonic gut (even if minor amounts might have been distributed in the body) and the administration of the dye after the closure of the vitelline duct did not result in a visible accumulation in the embryo.

It would have been a great advantage if radioactively labelled trypan blue had been available. The possibilities of discerning microscopically small quantities of the dye in the tissues are strongly limited. The autoradiographic technique which offers a comparatively high degree of sensitivity would have revealed whether a small amount of radioactivity was distributed also to other tissues than the gut, and if it was possibly retained in the tissues for a long period after the administration of the radioactive dye.

The previous failure in showing autoradiographically the presence of ^{14}C-trypan blue in the embryo (Wilson et al. 1963) might be due to the solubility of the dye in the histological solutions used. When the tissue sections examined in the present investigation were

fixed in a formaldehyde solution, most of the dye disappeared from the embryos. As the gut is a minor part of the whole embryo, a quantitative measurement of the radioactivity in *all the embryo* (performed by the same authors) is also likely to give low values, especially at the long time intervals used (1-12 days after the administration). The histological procedure may also be responsible for the predominantly negative results concerning trypan blue in the embryos in other investigations (Barber & Geer 1964; Greenhouse et al. 1969). The result of the present study, however, is supported by the report of Davis & Gunberg (1968) where trypan blue colouration was also seen in the gut (hindgut).

After the injection of single doses of trypan blue into pregnant animals, the highest teratogenic effect in the rat occurred when the dye was administered on the 9th day (according to the way of determining the pregnancy stage used here) (Wilson et al. 1959) and in the mouse on the 8th day (Schlüter 1970). A significant but lower teratogenic effect was seen up to the 11th and 10th days respectively. The disappearance of teratogenic effects thus seems principally to coincide with the blocking of the trypan blue transfer to the embryo via the vitelline duct found in this investigation. The fact that the teratogenic potential is higher at an earlier developmental age probably depends on the more undifferentiated state at that time. According to Beck et al. (1967) the decreased teratogenic potency is due to the fact that the chorioallantoic placenta takes over the transportation function which has previously been handled by the visceral yolk sac. There is no evidence, however, that this happens so early. It seems rather that the passage through the chorioallantoic placenta begins somewhat later and, what is more essential, that it only gradually increases (see chapter IV).

The direct effect of trypan blue on the embryo may be a good explanation of its teratogenic action. The theory of intraembryonic action is supported by the finding of Turbow (1965) that trypan blue is a teratogen when injected into cultured embryos of the rat *after* the time period stated by Wilson et al. (1959).The dye has produced reticuloendothelial tumours in the rat (Gillman et al. 1949; Gillman et al. 1973). It is possible that the teratogenic activity can be related to these oncogenic properties. If the action is in the embryo itself then it may be a direct one on the tissue to be damaged possibly by small amounts of the teratogenic principle transferred there from the gut. Another possibility is injury to the gut itself which may have a

profound influence on the whole embryonic development. Firstly, the gut takes part in the formation of many internal organs, secondly, it may influence other tissues by tissue interaction in their formation. The gut may also play a role in the transportation of material into the embryo from ingested amniotic fluid in later gestation, and such a function can be disturbed by a dye accumulation.

Schlüter (1970) showed that trypan blue had an effect on fetal resorption and retardation rates in the mouse from day 6 and up to day 14 of pregnancy, i.e. much later than the teratogenic effect was exerted. This effect may be mediated via the placental structures. The influence on the function of the visceral yolk sac probably is essential, as the dye is accumulated there from the time of its formation throughout pregnancy. It therefore is tempting to make a distinction between the etiology of malformation and that of resorption and retardation. They may not necessarily be regarded as expressions of the same damage. This question is of more universal significance in experimental teratology (see Wilson 1973).

CHAPTER III

INORGANIC MERCURY: CHANGES IN THE EMBRYONIC-FETAL UPTAKE THROUGHOUT GESTATION RELATED TO PLACENTAL DEVELOPMENT

The discussion about embryonal and fetal toxicity of mercury has been focused on the organomercurials, especially methyl mercury. Among mercury compounds this latter substance is important as a food contaminant and it is regarded as the cause of the fetal Minimata disease (Murakami 1972). The report of Murakami summarizes the experimental studies on its teratogenic effects in small laboratory animals. It shows that the organic mercurials are teratogenic during the entire organogenetic period and cause fetal brain damage in late gestation. Comparisons of the placental transfer of methyl mercury and inorganic mercury have revealed that the more lipid soluble methyl mercury is much more readily transferred to the fetus (Berlin & Ullberg 1963 a; Mansour et al. 1974).

It has been shown that inorganic mercury is teratogenic in the golden hamster in the early stages of organogenesis (Gale & Ferm 1971; Gale 1974). Combined treatment with cadmium and inorganic mercury causes peculiar variations in the fetal damage depending on the relative order of their administration (Gale 1973). Zinc affects the teratogenesis of both mercury and cadmium (Ferm 1972; Gale 1973). These interesting interactions indicate that cadmium and mercury at some level have a common mechanism of damage to the embryo, even if the final effects on the embryo differ.

Cadmium and mercury show some similarities in their behaviour in the organism. They both have an affinity for the sulfhydryl groups, and are strongly bound to the serum proteins (Hughes 1957; Passow et al. 1961; Nordberg 1972). They show a long term retention in the body, especially in the kidney. It was therefore found relevant to study whether there are similarities in the embryonal uptake of inorganic mercury after implantation to that previously found for cadmium (see chapter I). As earlier studies on the transplacental passage of inorganic mercury in late gestation (Berlin & Ullberg 1963 a; Garrett et al. 1972) indicated that it was low, it was considered of interest to follow the changes in the embryonic-fetal uptake of the metal as a function of the pregnancy stage. Autoradiography and gamma scintilla-

tion spectrometry were used to study these conditions in hamsters and mice. Determinations of the total fetal weight, dry weight percentage and the protein content in the fetal and maternal tissues at the different stages of gestation were done to possibly correlate the fetal concentration of mercury with the changes in these various parameters.

MATERIALS AND METHODS

Isotope

$^{203}HgCl_2$, spec. act. 0.50 μCi/μg and 1.61 μCi/μg ^{203}Hg respectively, dissolved in distilled water, was obtained from The Radiochemical Centre, Amersham, England.

Animals

Three months old mice of the C57BL strain weighing 20-35 g and golden hamsters weighing about 100 g were used in this investigation. The environmental condition and mating procedure were described in chapter I.

Autoradiography

At the different days indicated in table III:1 the animals were injected as described in chapter I. Each mouse received 17.5-20 μCi $^{203}HgCl_2$ in 50 μl (corresponding to 0.4-1.7 μg Hg per gram body weight) and each hamster 100 μCi in 300 μl (corresponding to 0.60-2 μg Hg per gram body weight). After predetermined intervals the animals were killed, autopsied, embedded, sectioned and autoradiographed as described in chapter I. Mice on day 12 or later and the hamster on day 14 of pregnancy were frozen and sectioned intact. The autoradiographic exposure time ranged from 5 to 30 days.

Impulse counting

Mice bred as described before received intravenously 3 μl per gram body weight of a water solution of $^{203}HgCl_2$ containing 64 μg ^{203}Hg/ml (0.192 μg or 0.309 μCi per gram body weight). Seven animals were injected on day 12, 3 on day 13 and 14, 5 on day 15, and 4 on day 18 - respectively between 7.00 and 8.00 hours in the morning and

were then deeply anesthetized with ether 8 hr later. The pleural cavity was opened and 0.7-1 ml blood was collected in a syringe from the right heart ventricle and ejected into a centrifuge tube. The uterus was removed and washed in physiological saline to be cleansed of blood. Thereafter the uterine wall was cut through the antimesometrial decidua and the amniotic cavity was opened. In this way the fetus welled out and could immediately be removed by a clean forceps. The fluid surrounding the fetus was soaked up by a piece of gauze, the fetus was placed on a preweighed piece of aluminium foil and instantly weighed. A piece of liver and the left kidney were also removed and weighed in the same way. All the specimens were placed in plastic vials. The blood was centrifuged at 3,800 g and 100 or 200 µl of the serum were poured into plastic vials. All the samples were then radioassayed in a gamma spectrometer (Packard Auto-Gamma Spectrometer). The counting efficacy was about 14 per cent.

Determination of fetal weight

In the several experiments described in this monograph, mouse fetuses at different days of gestation have been removed from the uteri, thoroughly dried with gauze and immediately weighed for the different determinations. The mothers have been untreated or injected with tracer doses of radioactive isotopes which were presumed not to affect fetal development. All experiments have been performed between 12.00 and 16.00 hours on the respective days, and the stage of pregnancy was determined as described earlier. The mean fetal weight on the different days has been calculated.

Determination of dry weight percentage

Fetuses at different ages were removed from the uteri, weighed and dried in a vacuum evaporator at 40°C. They were then reweighed immediately after they had been taken out at room temperature to diminish errors due to the uptake of atmospheric moisture into the tissues. Maternal liver, kidney and muscular pieces were also treated in this way. The dry weight was expressed as a per cent of the wet weight.

Protein determination

The protein content of the fetuses at the different ages was measured with the Folin phenol reagent after alkaline copper treatment as described by Lowry et al. (1951). For this purpose fetuses were collected: 3 on day 12, 5 on day 14, 15 and 17 respectively, and

TABLE III:1. Injection regimen of $^{203}HgCl_2$ into pregnant golden hamsters and mice and the main subsequent distribution in the embryos and fetuses.

Day of gestation on which ^{203}Hg injected	*Interval between conception and injection* Days	Hours	*Interval between injection and autopsy* Hr	*Embryonic-fetal uptake* gut (specific)	all tissues
Hamster					
8	7	17	8	+	((+))
8	7	17	24	+	-
8	7	17	96	-	+
8	8	2	12	+	((+))
9	8	12	8	+	((+))
9	8	17	4	+	((+))
9	8	19	8	?	((+))
10	9	16	4	-	-
11	10	12	8	-	((+))
14	13	16	4	-	+
Mouse					
8	7	13	4	(+)	-
8	7	13	24	(+)	-
8	7	22	8	+	((+))
9	8	8	8	+	((+))
9	8	12	4	++	-
9	8	12	24	(+)	(+)
9	8	15	8	+ (2 embr.)	-
10	9	12	4	-	-
11	10	8	8	-	-
12	11	8	8	-	-
13	12	8	8	-	(+)
14	13	8	8	-	(+)
15	14	8	8	-	(+)
16	15	8	8	-	+
17	16	8	8	-	+

4 on day 18. They were then weighed, placed in water and frozen until used for the determinations. Four pieces of 2 maternal livers, kidneys and muscles were treated in the same way to get a reference for the fetal determinations. All tissues were thawed and homogenized in a Potter-Elvehjem glass homogenizer. Samples of these homogenates were used for the protein determinations. The measurements were made in a Perkin-Elmer Spectrophotometer 124. Working standards were prepared from bovine albumin.

Statistics

In the text, tables and figures, numerical values are given as a

mean ± standard error of the mean (S.E.). For evaluation of the differences between means, the one way analysis of variance was used. It was used to test the mean differences in the tissue/maternal serum distribution ratio for the maternal livers and kidneys and the homogenized fetal tissues respectively on the different days of gestation. Concerning the distribution ratio between the fetal tissues and the maternal serum this could be expected to vary in two ways. There could be firstly a variation between the different dams on the same gestational day due to biological differences and secondly, a variation between dams being pregnant on different days of gestation. Both these causes of variation were tested with the one way analysis of variance. As there is doubt whether the mother or the single fetus is the proper sampling unit for statistical evaluation (Becker 1974; Kalter 1974; Palmer 1974; Staples & Haseman 1974; Weil 1974), the analysis between different gestational days was performed in two ways - either based on the single fetus or the mean of all the fetuses within each mother as the sampling unit.

RESULTS

Autoradiography

The overall distribution pattern of $^{203}HgCl_2$ in the maternal body of both the mice (fig. III:1) and hamsters was similar to that found earlier (Berlin & Ullberg 1963 a). The highest concentration was seen in the renal cortex, followed by the visceral yolk sac endoderm (in late gestation), epiphyseal lines of the bones, intestinal epithelium, decidua basalis, liver, chorioallantoic placenta, bone marrow and blood. The blood-brain barrier to the mercury was effective.

Embryo, fetus. The principal findings concerning the embryonic-fetal uptake are summarized in table III:1. At the earliest gestational age studied in the mouse (7 days 13 hours - 4 hr survival time) no radioactivity was detected in the ecto- and mesodermal layers (fig. III:2). A weak uptake was seen in the embryonic endoderm below these layers in the antimesometrial pole of the conceptus. In the extra-embryonic endoderm and what seems to be the foregut and hindgut areas of the embryonic endoderm, a heavy accumulation was however observed.

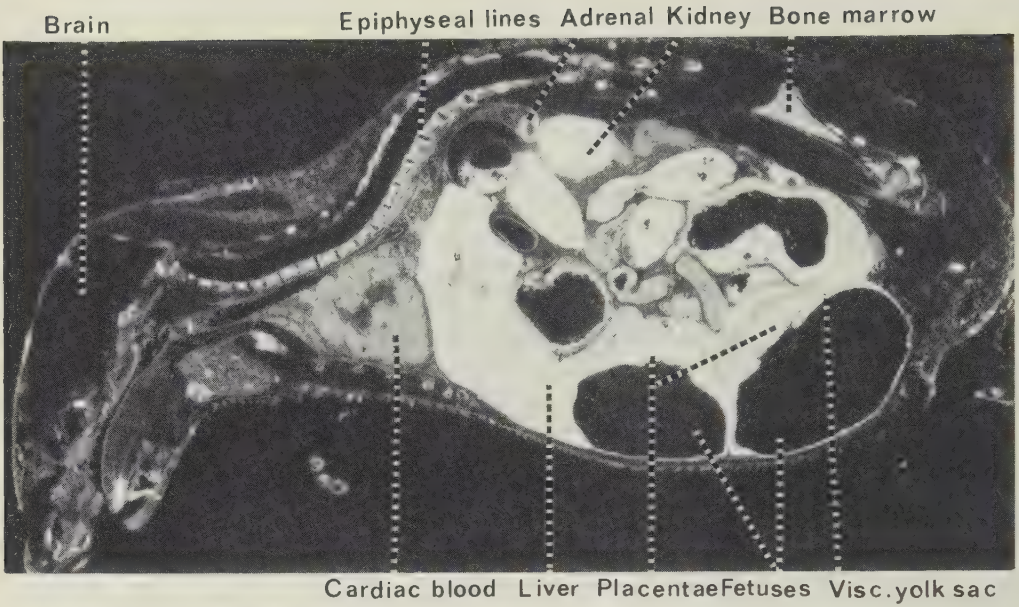


Fig. III:1. Whole body autoradiogram of a mouse at day 16 of gestation, 8 hr after injection of $^{203}HgCl_2$. High activity (white areas) is seen in the kidney, liver, epiphyseal lines and bone marrow, and a moderate concentration in the blood. No activity can be seen in the brain. In the uterus, high activity is seen both in the visceral yolk sac endoderm, chorioallantoic placenta and decidua basalis, but a low activity in the fetuses.

Twenty-four hours after the injection at the same gestational stage in both the mouse and the hamster, a rather high concentration of radioactivity was seen in the embryonic gut (fig. III:3). Injection after the gestational ages beginning with organogenesis and the invagination of the embryonic endoderm - forming the gut - resulted in mercury accumulation in the gut. This accumulation occurred up to the stage of the vitelline duct closure. Thus it was seen as late as the injection at 8 days 15 hours, but not after the injection at 9 days 12 hours in the mouse. In the hamster it was seen at 8 days 17 hours, only very weakly at 8 days 19 hours, and not at all later.

When compared with cadmium (see chapter I) the accumulation of mercury in the embryonic gut was lower relative to the other structures, e.g. the visceral yolk sac endoderm. Another difference was that small amounts of mercury passed to other structures besides the gut. This was seen in a greater concentration a long time after the

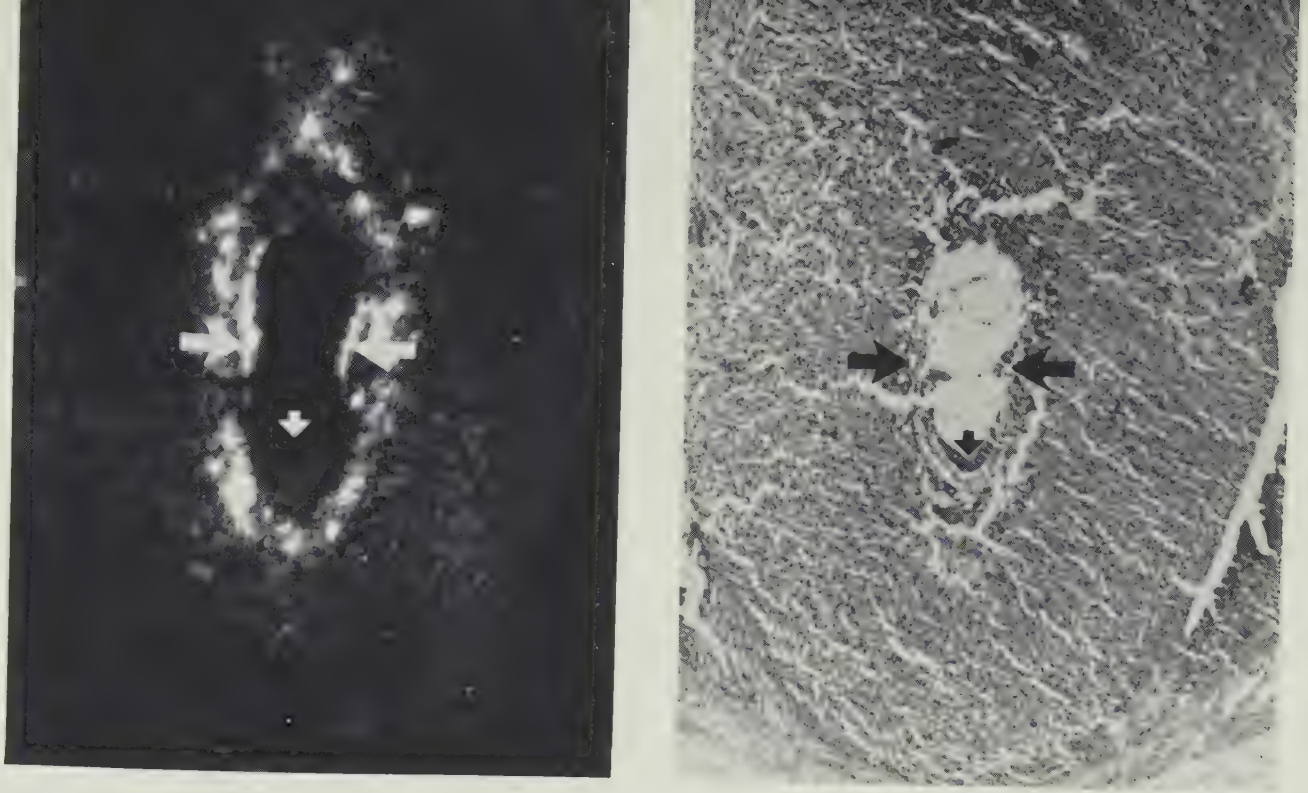


Fig. III:2. Autoradiogram (left) and the corresponding section (right) of a part of a mouse uterus 4 hr after the injection of $^{203}HgCl_2$ at 7 days 13 hours of gestation. The embryonic ecto- and mesoderm (small arrow) are void of radioactivity. In the proximal endoderm (large arrows) there is however a high activity. It seems probable that the foregut and hindgut areas are at the level of these arrows. The spotty pattern around the conceptus probably represents uptake in the trophoblasts (× 33).

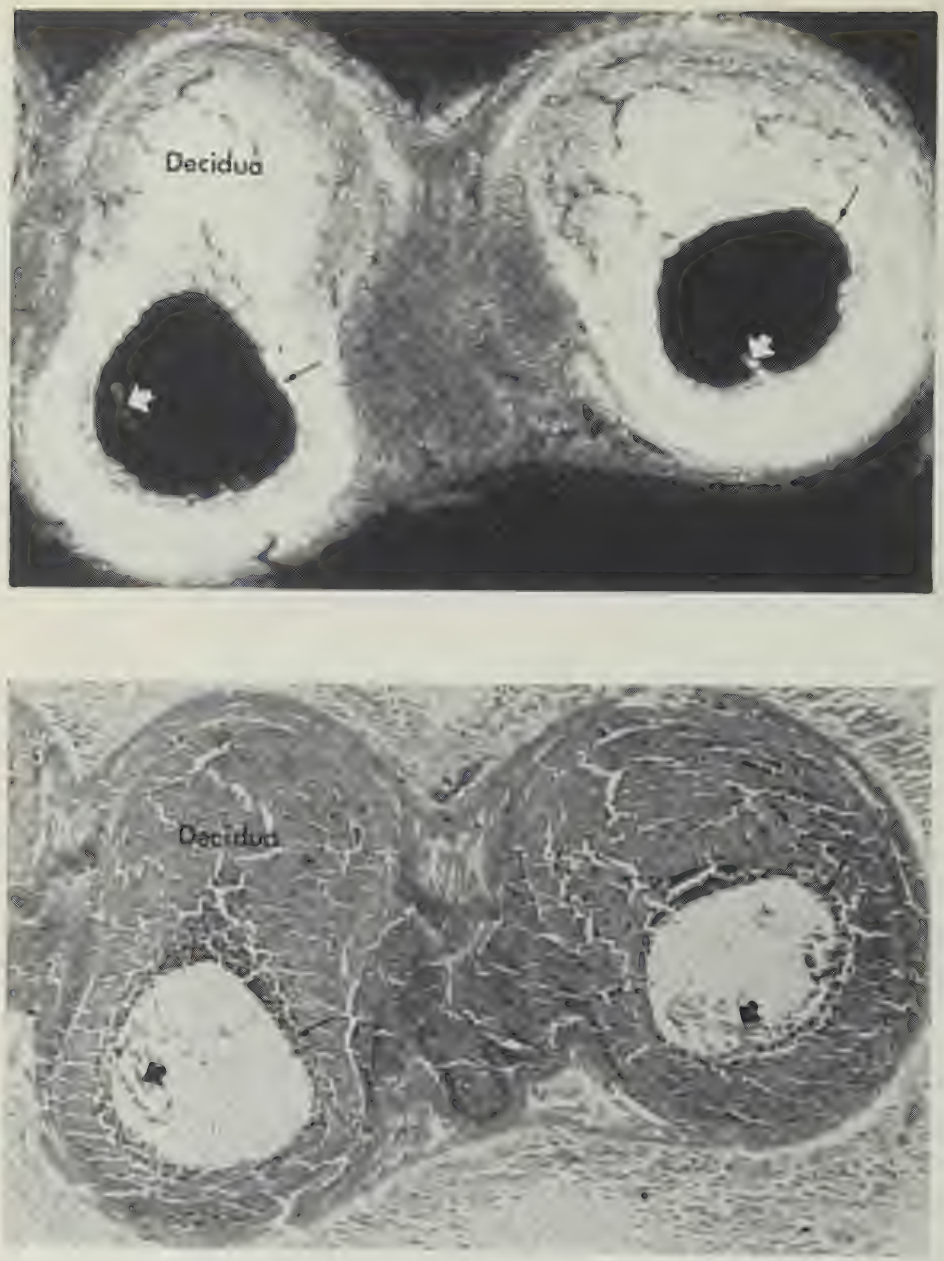


Fig. III:3. Autoradiogram (upper) with the corresponding section (lower) of a part of a hamster uterus 24 hr after the injection of $^{203}HgCl_2$ at 7 days 17 hours of gestation. Thick arrows show uptake in the gut of two different embryos. Thin arrows show the high uptake in the visceral yolk sac endoderm. There is also a high concentration in the surrounding decidua (× 11).

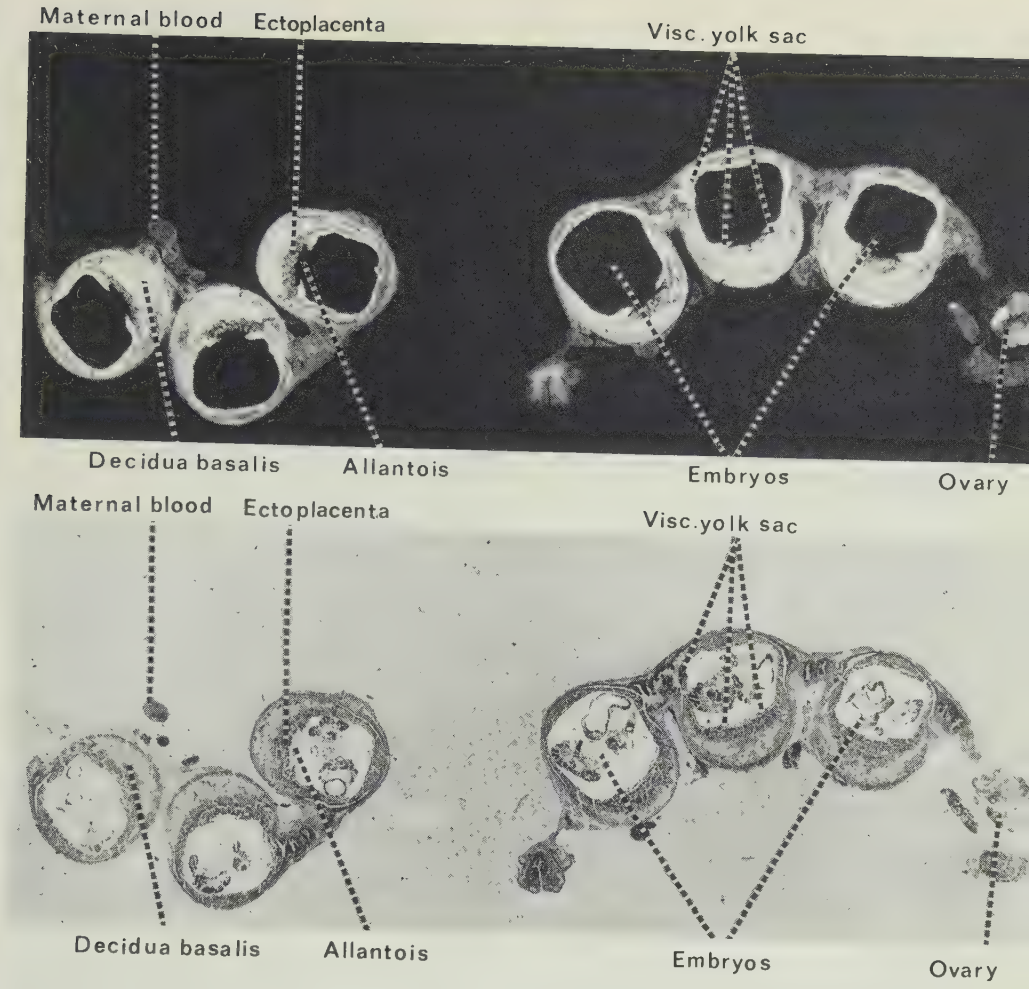


Fig. III:4. Autoradiogram (upper) and the corresponding section (lower) of a mouse 8 hr after injection of $^{203}HgCl_2$ at 10 days 8 hours of gestation. The embryos are void of radioactive mercury. A high accumulation (compared to the maternal blood) can be seen in the visceral yolk sac endoderm and in the decidua. In the ectoplacenta the concentration is comparable to the maternal blood. Note also the high concentration in the follicles of the ovary, and a lower concentration in the corpora lutea (× 3).

injection. A third difference was found in the hamster injected at 7 days 17 hours which was allowed to live for 96 hr, where a weak but visible uptake of mercury was found in all the fetal structures. In the corresponding situation cadmium was seen only in the alimentary tract where it most probably had been taken up during the hours following the injection. The mercury found in these late fetuses had probably been transported there continuously.

In the mouse embryos of the dams injected at 9 days 12 hours, 10 days 8 hours (fig. III:4) and 11 days 8 hours no radioactivity could be detected as judged by the blackening of the films. In the hamster embryos after the injection at 9 days 16 hours only very weak traces were seen.

From day 12 and up to day 17 (fig. III:5) an increasing, but still not high fetal uptake was seen in the mouse. Even at the latest gestational age the fetal concentration was low, about equal to that of the maternal muscular tissues. The fetal blood concentration was much below that of the maternal. In contrast to the maternal kidney, the fetal one did not accumulate mercury more than the other tissues and the distribution picture was very even. In the liver a spotty pattern of uptake was seen. There was a tendency for a blood-brain barrier against the mercury similar to that found in the mother. In the hamster there was a higher uptake of mercury in the embryos at 10 days 12 hours than those at 9 days and even higher at 13 days 16 hours. In the oldest hamster fetuses an uptake was seen especially in the cartilage.

Placental structures. In the visceral yolk sac endoderm there was a high accumulation already at the earliest gestational stage (figs III:2-4). This accumulative ability was seen throughout the pregnancy (fig. III:1) and seemed to increase with the gestational age.

Before the development of the chorioallantoic placenta the concentration in the ectoplacenta was comparatively low, and no activity could be detected in the allantoic part (fig. III:4). The concentration

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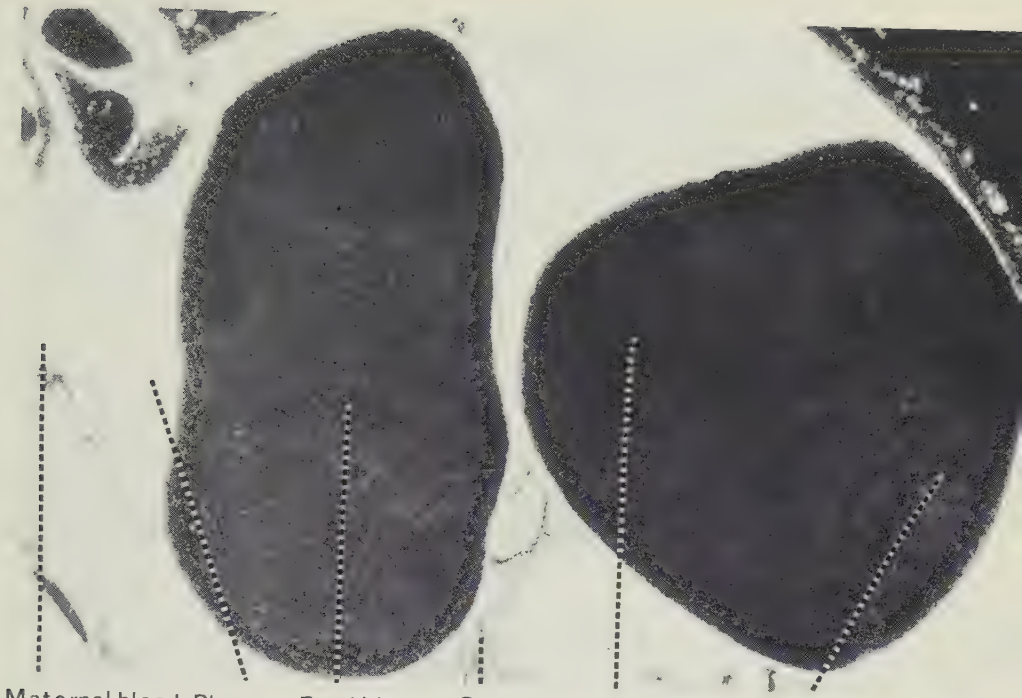
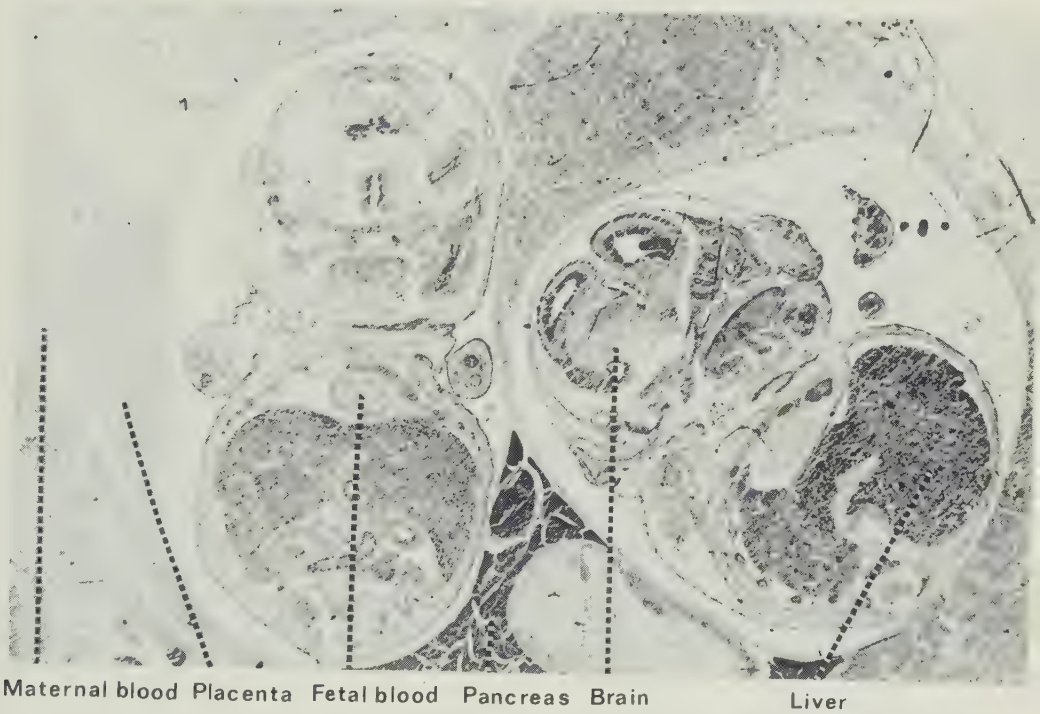
Fig. III:5 (on the opposite page). Detail of whole body autoradiogram (upper) and the corresponding section (lower) showing two mouse fetuses on the 17th day of gestation, 8 hr after the injection of $^{203}HgCl_2$ to the mother. There is a very high concentration ratio between the maternal and the fetal blood. In the liver there is a spotty uptake. In the brain the concentration is low (× 5).


Maternal blood
Placenta
Fetal blood
Pancreas
Brain
Liver


Maternal blood
Placenta
Fetal blood
Pancreas
Brain
Liver

in the chorioallantoic placenta increased with the gestational age. At 15 days 8 hours the placental concentration was almost equal to that of the liver (fig. III:1).

As was found for cadmium, also mercury accumulated in the decidua of the hamster in the early stages (fig. III:3). In the ovaries of both the mouse and hamster the follicular wall strongly accumulated mercury (fig. III:4), but in contrast to cadmium, its concentration in the corpora lutea was lower.

Impulse counting

The purpose of this impulse counting experiment was to determine an expected increase in the fetal concentration (compared to the maternal serum) with an advancing stage of gestation. Such a situation was probable, judged from the autoradiographic investigation, although the fetal concentration was comparatively low throughout gestation. Therefore, as the dams in all stages received the same dose per weight unit, and their body weight increased from about 22 grams on day 12 to about 30 grams on day 18, the increasing fetal contribution to the maternal weight was expected to increase the maternal serum concentration of radioactivity in late gestation. Such a tendency was observed but the individual variations were more pronounced.

The rate of transfer of chemicals to the fetus is primarily due to the concentration gradient between the maternal and fetal serum. From this point of view it was found relevant to state the fetal uptake as the radioactivity distribution ratio between whole fetuses (based on radioactivity per gram tissue) and the maternal serum (radioactivity per ml). This was done to possibly decrease uncontrollable errors as to method of administration, the biological variations as to clearance of the substance from the blood, the metabolism, the excretion etc.

In table III:2 the radioactivity distribution ratio between the maternal liver and kidney respectively and the serum can be seen. The liver/serum ratio was fairly constant throughout gestation, while the kidney/serum ratio varied within wide limits but without an obvious trend, and the variation between the different days was not statistically significant.

In table III:3 the radioactivity distribution ratio between the homogenized fetal tissues and the maternal serum is shown. The values of the two columns do not differ essentially from each other. There is a highly significant difference between the ratios of the different

TABLE III:2. Radioactivity distribution ratio between liver and kidney (per gram) respectively, and serum (per ml) 8 hr after the intravenous injection of $^{203}HgCl_2$ in pregnant mice at different days of gestation. Each value represents the mean ± S.E. with the number of animals in brackets.

Day of gestation	*Liver*	*Kidney*
12	4.0 ± 0.3 (7)	10.6 ± 2.5 (7)
13	4.3 ± 0.1 (3)	12.6 ± 2.0 (3)
14	4.1 ± 0.4 (3)	9.3 ± 1.4 (3)
15	4.1 ± 0.2 (5)	6.9 ± 0.5 (4)
18	4.9 ± 0.5 (4)	12.8 ± 2.1 (4)
Significance level	$p>0.05$	$p>0.05$

days ($p<0.001$). In fig. III:6 the radioactivity distribution ratio between fetal tissues and maternal serum (table III:3, left column) is shown graphically, and simultaneously the ratio mean of each mother is indicated to demonstrate the distribution of values within each day of gestation. It is interesting to note that there is also a statistically significant difference between the mothers on the same day of gestation. This may partly be due to differences in placental maturation.

Determination of fetal weight

The mean fetal weight of the mice increased between day 12 and day 18 from 34 mg to 890 mg, which is a 26-fold increase (fig. III:7). The increase was not linear but accelerated throughout the pregnancy.

Determination of dry weight percentage

A change in dry weight (expressed as a per cent of wet weight) of the mouse fetuses as a function of the gestational stage can be expected to influence the uptake of chemicals during the different stages. As can be seen in table III:4 there was about a 53 per cent increase in the dry weight percentage from day 13 to day 18 of gestation. This change was statistically significant ($p<0.001$). However, as late as on day 18, the fetal dry weight percentage was only half of that of the maternal liver.

TABLE III:3. Radioactivity distribution ratio between homogenized fetal tissues (per gram) and the maternal serum (per ml) 8 hr after the intravenous injection of $^{203}HgCl_2$ in pregnant mice at different days of gestation. Each value represents the mean ± S.E. with the number of sampling units within brackets. In the column to the left each fetus was used as the sampling unit[1]; to the right the mean of the distribution ratios of all fetuses from each mother was used as the sampling unit. The significance level for the comparison between mothers on the same day of gestation is given for the left column, and for the comparison between different days under each column.

Day of gestation	*Each fetus= =one sampling unit*	*p*	*Mean within the mother= =one sampling unit*
12	0.027 ± 0.001 (24)[1]	∿0.001	0.029 ± 0.003 (7)[1]
13	0.035 ± 0.001 (25)	<0.025	0.035 ± 0.003 (3)
14	0.038 ± 0.001 (25)	<0.001	0.038 ± 0.002 (3)
15	0.041 ± 0.002 (33)	<0.001	0.041 ± 0.004 (5)
18	0.067 ± 0.002 (25)	<0.001	0.066 ± 0.006 (4)
Significance level (between days)	p<0.001		p<0.001

[1]On day 12, each fetus from 3 mothers was counted separately, while those of the other 4 mothers were weighed and counted together. The number 24 in the first column therefore stands for 20 single fetuses and 4 samples with collected fetuses.

Protein determination

As well as the dry weight percentage, also the protein concentration of the fetal tissues may be a determining factor in the fetal accumulation of chemicals strongly bound to proteins, on the assumption that other factors do not negate its importance. Table III:4 shows that there was about a 96 per cent increase in the protein concentration in the fetuses from day 12 to day 18. This change was statistically significant ($p<0.001$). The fetal protein concentration on day 18 was, however, only about 44 per cent of that of the maternal liver.

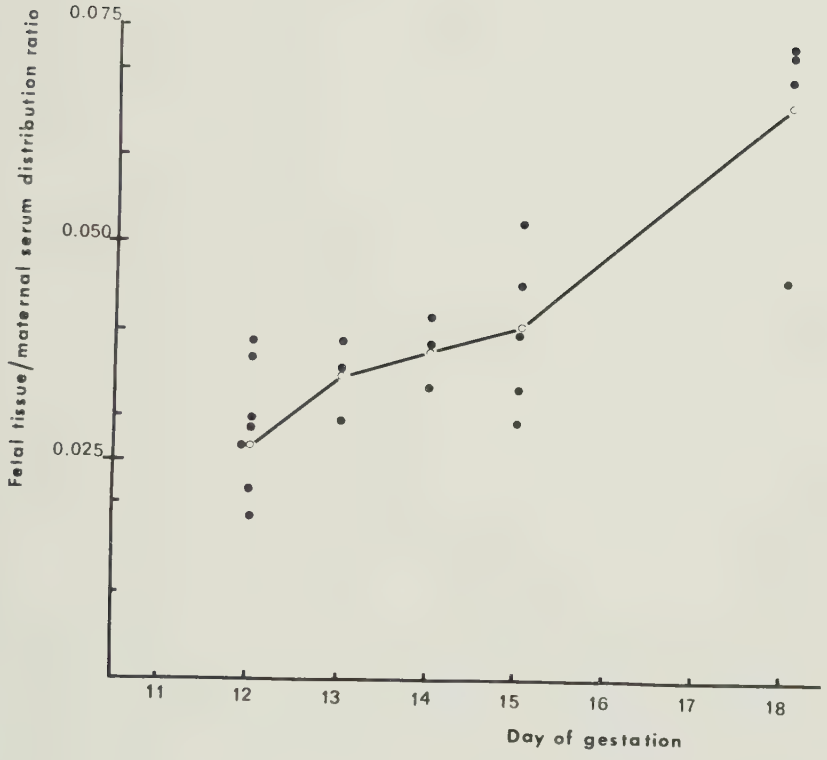


Fig. III:6. Variations in radioactivity distribution ratio between homogenized fetal tissues (per gram) and the maternal serum (per ml) as a function of the gestational stage in the mouse. $^{203}HgCl_2$ was injected 8 hr prior to sampling on the days indicated. The points indicate the mean ratio within each mother and the circles connected with lines indicate the means on the respective days based on each fetus as the sampling unit (table III:3, left column). There is a 2.5-fold increased ratio from day 12 to day 18.

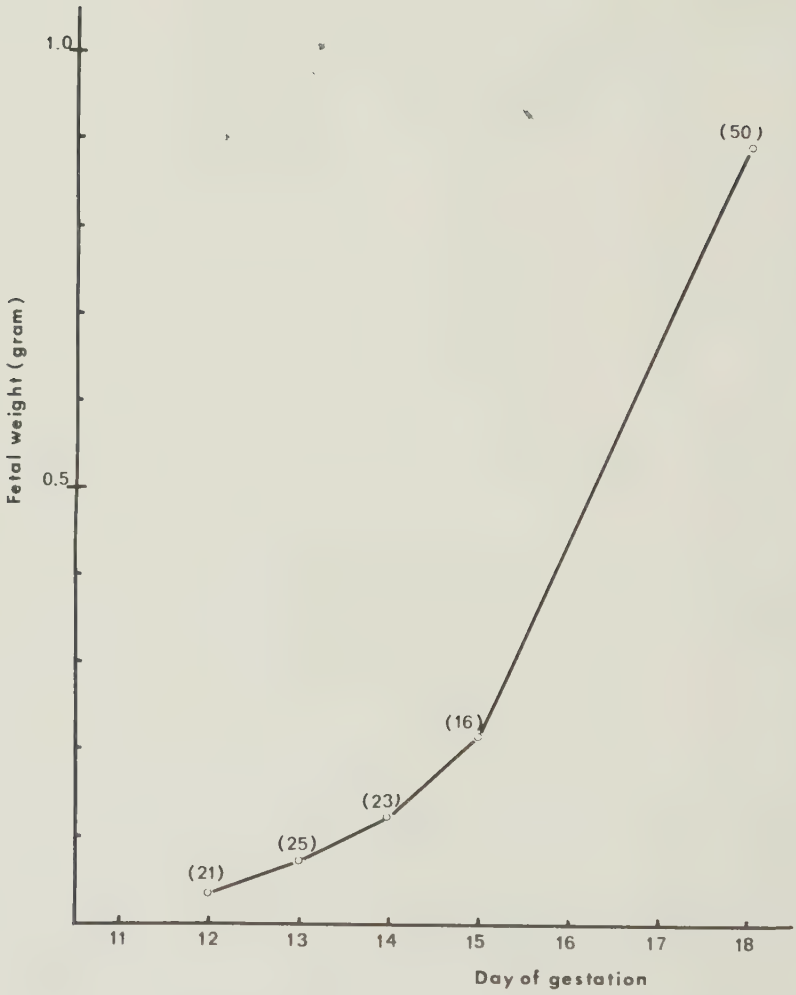


Fig. III:7. Variation in fetal weight from day 12 to day 18 in the mouse. Figures within brackets indicate the number of samples. The fetuses were collected between 12.00 and 16.00 hours on the respective day. The increase is not linear but accelerates with advancing gestation.

TABLE III:4. Dry weight and protein concentration determinations of selected maternal tissues and homogenized fetuses at different developmental days. Dry weight is expressed as a per cent of wet weight and protein as mg per gram wet weight. Mean ± S.E.

Tissue		*Dry weight* (per cent)	*Protein* (mg/g)
Maternal liver		30.4 ± 0.4 (11)	216 ± 18 (4)
kidney		25.9 ± 0.4 (8)	207 ± 12 (4)
muscle		26.6 ± 0.8 (10)	216 ± 11 (4)
Fetus (day of gestation)	12		49 ± 4 (3)
	13	10.1 ± 0.2 (8)	
	14	9.6 ± 0.1 (5)	72 ± 1 (5)
	15		69 ± 2 (5)
	17	12.8 ± 0.3 (5)	98 ± 2 (5)
	18	15.5 ± 0.9 (7)	96 ± 4 (4)
Significance level (fetuses) (between days)		$p<0.001$	$p<0.001$

DISCUSSION

This study has shown autoradiographically a change in the fetal concentration of injected inorganic mercury ($^{203}HgCl_2$) in mice and hamsters as a function of the maternal gestational age. In the early organogenetic period a significant accumulation was observed in the embryonic gut, and in some animals traces were found in other embryonic structures. With vitelline duct closure a period began during which no mercury could be detected in the embryos, but later in gestation an increasing fetal concentration was observed. An impulse counting experiment during the last third of pregnancy confirmed the autoradiographic results since it showed that 8 hr after the intravenous injection of mercury the distribution ratio between the fetal tissues and the maternal serum steadily increased.

Early gestation

The previous experiment with cadmium (see chapter I) showed that this metal accumulates in the embryonic gut wall during a restricted period of embryonic development - when it is also teratogenically active according to Ferm and co-workers (Ferm & Carpenter 1967; Ferm 1971; Gale 1973; Gale & Ferm 1973). The present result which showed that inorganic mercury accumulated in the gut wall similarly to cadmium may perhaps explain the interesting finding that mercury and cadmium interfere with each other in their embryotoxic action (Gale 1973). Thus, simultaneous injections of cadmium and mercury increased the fetal resorption rate, and this effect was much more pronounced when cadmium was injected one hour before the mercury than vice versa (Gale 1973). The interpretation may be that these two metals have a common "receptor" in the embryo. If cadmium, which is the by far most embryotoxic of the two metals according to Gale (1973), gets first access to the presumptive receptor, the damage will be more severe than if the less embryotoxic mercury is first bound and possibly hinders the cadmium binding to some degree. According to the present result this "receptor" may be in the gut. It is also possible that an earlier injection of cadmium may change the barriers in the gut and visceral yolk sac, increasing their permeability to mercury and consequently the fetal concentration of this metal.

As was discussed in chapter I, the mechanism of damage to the embryo from cadmium accumulation in the gut is not readily explained, and this is true also for mercury. There may be other explanations of its teratogenicity. Mercury was found to be less effectively blocked than cadmium in its transfer to embryonic tissues other than the gut. For example, one of the hamsters injected with mercury at 7 days 15 hours which was autoradiographed 96 hr later showed no intestinal retention as high as cadmium, but had detectable concentrations in all tissues, probably transferred there continuously. Therefore a direct effect on embryonic tissues sensitive to damage is possible.

Both the embryotoxic and the more specific teratogenic effects of mercury and cadmium, alone or in combination, may however depend on their common influence on extraembryonic structures and functions. Both are heavily accumulated in the visceral yolk sac placenta which is believed to play an essential role in early embryonic nutrition (see e.g. Beck et al. 1967). If maternal factors are considered of significance in the induction of embryonic defects, the similarities in the biochemical behaviour of mercury and cadmium on other levels

may explain their interaction.

Second half of gestation

After vitelline duct closure there was both qualitatively and quantitatively a change in the uptake of mercury in the embryo. On the 10th and 11th days no essential uptake could be detected. Later and throughout gestation a steady increase in fetal concentration was observed. Actually there was a 2.5-fold increase in fetal concentration between day 12 and 18. Taking into consideration the simultaneous 26-fold increase in fetal weight (see fig. III:7) - the average increase in the amount of mercury transported to the fetus was 66-fold. Schulert et al. (1969) who measured the fetal concentration of mercury in rat embryos, found a reduction in the fetal uptake from day 14 to day 20. A later report from the same laboratory showed results similar to those found in the present investigation (Mansour et al. 1974).

To make possible a relevant discussion of the reasons for this increasing fetal concentration, it is essential to understand in which form the inorganic mercury may be transferred. According to Hughes (1957) the extremely large association constant for the reaction between mercury and the thiols must mean that all but traces of the mercurials will be bound to the thiols. Five to ten per cent of the total thiol content of human plasma is present in the form of smaller molecules. At the toxic blood level of mercury there is up to 8,000-fold excess of sulfhydryl groups over the available mercury (MacGregor & Clarkson 1974). Passow et al. (1961) stated that less than 10 per cent of intravenously injected mercury ($HgCl_2$) was ultrafiltrable. Clarkson (1972), reviewing several investigations, summarized that mercury in the serum was bound mainly to the albumin and γ-globulin fractions and that less than 1 per cent was diffusible.

Taking these facts into consideration it seems reasonable to assume that only minute amounts of nonbound mercury are available to be transported through the placenta. If such mercury reaches the placenta it is probably more inclined to bind e.g. to the numerous placental enzymes than to be transferred to the fetal circulation (note the high accumulation in the placenta, fig. III:1). It seems more probable that the mercury bound to sulfhydryl groups on diffusible complexes is transferred. On the other hand larger proteins like IgG, albumin and labelled whole serum (to which mercury can be bound) have also been shown to be transported to the fetus (Mayersbach 1958; Brambell 1966; Morphis & Gitlin 1970; Hemmings 1974). In fact

Morphis & Gitlin (1970) showed an increase in the uptake of human albumin into the mouse fetus from day 11 to day 20 of gestation, which rather paralleled that of mercury in the present study. The fact that the more lipid soluble methyl mercury however is transferred through the placenta much more readily than inorganic mercury probably means that the chemical properties of the original molecule are not completely eliminated.

Theoretically there are at least three principally different factors that may be responsible for the change in transport with gestational age.

1. *Changes in the physico-chemical state of mercury in the maternal and fetal serum.* As can be seen in chapter IV (discussion) changes in the serum protein and hydrogen ion concentration on both sides of the placental barrier may change the rate of placental transfer. According to the argumentation presented above these factors are probably of minor importance in the mercury transfer.

2. *Changes within the embryo.* The comparison of the distribution pattern of mercury in the fetus to that of the mother shows that it is far more even in the fetus. This undoubtedly reflects a relatively lower concentration in the fetal organs of the constituents active in the binding of the mercury in e.g. the maternal liver and kidney and this fact may partly explain the low fetal concentration of mercury. As mercury is supposed to bind preferentially to proteins (Passow et al. 1961) the determination of the protein concentration was performed. The mixed fetal tissues had a low protein concentration compared to the maternal liver, kidney and muscles, but the concentration increased significantly with maternal gestational age. The findings may partly be an explanation of the low but - with advancing gestational age - increasing accumulation of mercury in the fetal tissues. However the probable abundance of the thiol groups compared to mercury in the fetus as in the maternal serum (discussed above) probably rejects also this hypothesis.

3. *Changes in placental permeability.* The most probable interpretation of the finding under discussion is that there is a low, but increasing placental permeability to mercury (and probably also other compounds; see especially chapter IV). Fig. III:5 shows the wide difference in the blood concentration of the mercury in the

mother and the fetus. This difference probably reflects a similar concentration difference between the maternal and the fetal serum.

In rodents two main routes of transfer to the fetus are commonly discussed: that of the visceral yolk sac placenta, and that of the labyrinthine chorioallantoic placenta. The mouse visceral yolk sac endoderm develops absorptive characteristics already on day 7-8 and blood vessels on day 9 (Rugh 1968; Haar & Ackerman 1971 a,b). The chorioallantoic placenta and the allantoic circulation is established by day 10-11 (Rugh 1968; Theiler 1972). The visceral yolk sac placenta may therefore be expected to contribute to embryonic nutrition and possibly to transfer of other chemicals some days earlier than the chorioallantoic placenta. The fact that only traces of injected mercury could be seen in the mouse embryos from the time of vitelline duct closure and up to day 12 means that the visceral yolk sac placenta does not transfer significant amounts of mercury during this time. It should be emphasized that there was however a heavy accumulation within the visceral yolk sac endoderm itself. The beginning of the allantoic circulation may be the explanation of the increasing fetal uptake from day 12 and subsequently.

It cannot be excluded however that there is an increasing visceral yolk sac transfer with advancing gestational age. Calarco & Moyer (1966) described the morphological features of visceral endodermal cells from day 12 to day 18. Their observations suggested that although synthesis and absorption doubtless occurred simultaneously throughout gestation, the emphasis was on synthesis during the first half of the period and absorption during the second half. They connected this change with the rupture of the Reichert's membrane (on the 15th day). The present investigation, however, has clearly shown that there is no dramatic change in the accumulation of the different chemicals in the visceral yolk sac that can be correlated to the rupture of the Reichert's membrane.

In the rabbit the γ-globulins and other serum proteins were transferred to the embryo and fetus at different stages of gestation via the visceral yolk sac (Smith & Schechtman 1962; review by Brambell 1966) and this was considered the only route for immunoglobulins to the fetus. Later it has been shown that also the chorioallantoic placenta of the rabbit transfers both rabbit and bovine IgG (Hemmings 1974).

Ferritin was readily taken up in the rat visceral yolk sac

endoderm from day 10 to 21 but signs of transfer to fetal vitelline blood vessels increased in late gestation (Lambson 1966). Seibel (1974), using horseradish peroxidase, found evidence of transport throughout this period. Robertson et al. (1971), studying mice of late gestation, showed a probable transfer of horseradish peroxidase through the visceral yolk sac but not the chorioallantoic placenta.

The rather extensive literature on visceral yolk sac placental transfer of homologous as well as heterologous macromolecules in different species partly reviewed here, does not give an answer to the question whether this placental structure may be responsible for the observed change in transfer of mercury (which is probably protein bound) in the present study. Also iron shows an increasing transfer to the fetus during the second half of gestation both in the rabbit and rat (Bothwell et al. 1958; Davies et al. 1959; Kaufman & Wyllie 1970, 1972) and is probably transferred via both of these placental structures - although most rapidly via the chorioallantois. It may however be inappropriate to compare the route of placental transfer of mercury to that of an essential metal.

It seems that the most probable explanations for the increasing mercury transfer with advancing gestational age are the increasing blood flow both in the maternal and fetal circulations of the chorioallantoic placenta, the increasing area of contact between the maternal and fetal vessels, and, maybe more essential, the progressive attenuation of the trophoblastic layers separating these two circulatory systems which has been found to occur (Franke 1969). Histological sections from the 11th day mouse placenta showed a sparsity of vessels and only a few close contacts between the maternal and the fetal blood vessels compared to the 18th day placenta (personal observation).

CHAPTER IV

THE HERBICIDE 2,4,5-T: EARLY PLACENTAL BARRIER AND ACCELERATED FETAL UPTAKE WITH ADVANCING GESTATION

The discussion concerning the teratogenic potency of the phenoxyacetic acid herbicides is well known. It has drawn fire from newspaper reports especially in Vietnam, the U.S.A. and Sweden on suspected involvement in human malformations. The first published report on experimentally induced malformations (in rats) using 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) appeared in 1970 (Courtney et al. 1970). The preparation used however contained 30 ppm of 2,3,7,8-tetrachlorodibenzo-p-dioxin (here called dioxin) which is known to be extremely toxic. Their results were therefore reevaluated in several investigations, where the teratogenic effects of 2,4,5-T, 2,4-dichlorophenoxyacetic acid (2,4-D) and dioxin alone or in combination were tested (Emerson et al. 1970; Courtney & Moore 1971; Roll 1971; Khera & McKinley 1972; Neubert & Dillmann 1972; Båge et al. 1973; Frohberg 1974). Although these studies have given contradictory results, it seems clear that 2,4-D, 2,4,5-T and the dioxin are all teratogenic and embryotoxic and that 2,4,5-T and dioxin act synergistically when administered together (cleft palate) (Neubert & Dillmann 1972).

In a previous autoradiographic investigation no radioactivity was found in the embryos at early gestation after the administration of ^{14}C-2,4,5-T, but in late gestation the fetal tissues reached a level similar to that of the maternal ones (Lindquist & Ullberg 1971). Fang et al. (1973) also recorded variations in the transplacental passage of 2,4,5-T, but the stage of pregnancy was unfortunately not specified.

The different phenoxyacetic acids have been found to be stable in the body (Levey & Lewis 1947; Khanna & Fang 1966; Erne 1966 b; Fang et al. 1973; Piper et al. 1973), only a few per cent of the excreted substance being metabolites.

The present study was undertaken to estimate the embryonic-fetal uptake of 2,4,5-T throughout gestation, with correlation to the placental changes and the different degrees of teratogenic sensitivity. Autoradiography and liquid scintillation spectrometry were used.

Labelled compound

The radioactive substance, 2,4,5-trichlorophenoxy(1-^{14}C)acetic acid (^{14}C-2,4,5-T), spec. act. 54 mCi/mmol (210 µCi/mg) and 56 mCi/mmol (218 µCi/mg) respectively, was obtained from The Radiochemical Centre, Amersham, England, dissolved in benzene. The benzene was evaporated by a nitrogen gas flow and the ^{14}C-2,4,5-T was redissolved in 50 per cent ethanol. The radiochemical purity was 95-99 per cent (thin layer chromatography on silica gel; solvent system: chloroform:cyclohexane:acetic acid 80:20:10).

Animals

Mice (C57BL) and golden hamsters were used. The environmental conditions and mating procedure were the same as those described in chapter I.

Autoradiography

Mice received 5 µCi or about 24 µg dissolved in 100 µl 50 per cent ethanol intravenously in a tail vein, and golden hamsters 10 µCi or about 48 µg in 100 or 200 µl 50 per cent ethanol in a jugular vein under light ether anesthesia. They were injected on different days of gestation according to the schedule seen in table IV:1. After predetermined times the animals were anesthetized to death and the uteri were removed and embedded. Only a mouse at day 16 of gestation was embedded for whole body autoradiography. Thereafter the tissues were sectioned and autoradiography was performed as described in chapter I. The exposure time ranged from 2 to 11 months.

Liquid scintillation counting

Mice on different days of gestation, from day 11 to day 18, were weighed (21-38 g) and injected intravenously in a tail vein, each animal receiving 0.1 µCi ^{14}C-2,4,5-T (dissolved in 3 µl 50 per cent ethanol) per gram body weight. This corresponds to about 0.5 µg per gram body weight. Four animals were injected on each day 11, 12, 14 and 18 respectively and 2 animals on day 16. Four hr later the animals were deeply anesthetized with ether and the procedures used for investigating inorganic mercury (chapter III) were repeated for the collection of the blood serum, pieces of the liver and renal cortex and the

embryos or fetuses. In three out of the four animals injected on day 11 several embryos were pooled so that from each mother only 2 fetal samples were obtained. All the embryos at day 16 and 18 of gestation were thoroughly homogenized in separate vials and samples (weighing about 100 mg) of these homogenates were used for impulse counting. 100 µl blood serum samples were used. The whole embryos or tissue samples were dissolved in Soluene 100®. After adding the scintillation fluid (4.9 g PPO + 0.1 g POPOP/liter toluene) the samples were counted in a Packard liquid scintillation spectrometer. Correction for quenching was made by the use of an external standard.

TABLE IV:1. Injection regimen of ^{14}C-2,4,5-T into pregnant golden hamsters and mice and the main subsequent distribution in the embryos and fetuses.

Day of gestation on which ^{14}C-2,4,5-T injected	*Interval between conception and injection*		*Interval between injection and autopsy*	*Embryonic-fetal uptake*	
	Days	*Hours*	*Hr*	*gut (specific)*	*all tissues*
Hamster					
8	7	17	4	++	-
8	7	17	24	-	-
8	7	19	6	++	-
9	8	12	6	-	-
9	8	17	4	(+)	-
10	9	16	4	-	-
11	10	12	6	-	-
Mouse					
9	8	8	4	++	-
9	8	8	12	++	-
9	8	12	4	++	-
9	8	20	12	+	-
10	9	8	8	(+)	((+))
11	10	8	8	-	(+)
12	11	8	8	-	+
13	12	8	8	-	++
16	15	8	8	-	+++++

Autoradiography

The whole body autoradiographic distribution picture (in the mouse on the 16th day of gestation; fig. IV:1) was in accordance with that found by Lindquist & Ullberg (1971). The highest activity was seen in the kidney and in the visceral yolk sac. The concentration in the blood was high, while it was lower in the liver and still lower in the muscular tissues. The blood-brain barrier to the 2,4,5-T was effective.

Embryo, fetus. The main findings of embryonic-fetal uptake are summarized in table IV:1. In the mouse embryos the concentration was very low in all the observed stages of gestation up to day 10. During this period (up to day 9) the embryonic gut communicated with the yolk sac cavity. In this cavity and also in the lumen of the gut a very high concentration of radioactivity was observed (fig. IV:2). It was not possible to determine whether there was any absorption of this material in the endodermal cells of the gut wall. In the visceral yolk sac endoderm (which at this stage had probably a structure similar to that of the gut endoderm) no accumulation was present. No embryonic structure or surrounding fluid retained radioactivity 24 hr after the injection at this stage of pregnancy in the hamster. In a few embryos of the mothers injected at these early stages traces of radioactivity could be detected in all the tissues, but more often the embryos had too low radioactivity to show up.

On day 11 the outline of the embryos was distinguishable due to the higher uptake in all the embryonic tissues when compared to animals injected at earlier stages of gestation. It was too low, however, to be visualized in the picture of fig. IV:3. At this stage there was not any higher uptake in the gut than in the rest of the embryo. On day 12 and even more on day 13 (fig. IV:4) the fetal concentration was higher, and within the embryo the distribution was even. On day 16 (fig. IV:5) the fetal concentration had increased considerably and equalled that of the maternal muscular tissue. Now a certain differentiation could be distinguished in the fetal distribution, e.g. a rather high concentration was seen in the blood and a low one in the brain.

Also in the hamster embryos a high uptake was observed in the

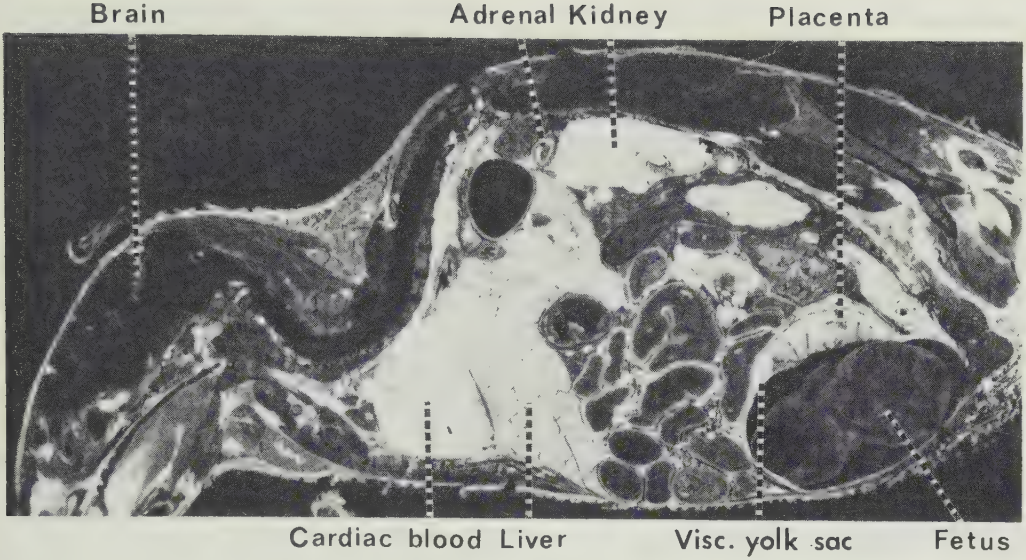


Fig. IV:1. Whole body autoradiogram of a mouse on day 16 of gestation, which was injected with ^{14}C-2,4,5-T 8 hr prior to death. The highest concentration is seen in the kidney, maternal blood and visceral yolk sac. It is moderate in the liver and rather low in the muscles. Radioactivity can be seen in the fetus (see also fig. IV:5).

gut lumen in the early stages. This was true for injections done up to 8 days 17 hours although only one embryo accumulated radioactivity. Twenty-four hours after the injection on day 8 only insignificant traces were found in the whole uterus. On day 10 and 11 no radioactivity was detected in the embryos.

Placental structures. In the uterus the concentration seemed to be at least partly dependent on the serum concentration as it was higher in the more spongious areas. In the yolk sac fluid of early gestation the concentration was very high (fig. IV:2), and also at day 16 a high concentration was seen in the uterine cavity close to the visceral yolk sac placenta (figs IV:1 and 5). This often concealed a possible accumulation in the visceral yolk sac placenta, but in areas where this was not surrounded by a high activity, its concentration was rather high from day 11 to day 16. The activity in the chorioallantoic placenta was always lower than in the blood (figs IV:1 and 5). The concentration of radioactive material in the amniotic cavity and in the exocoelom was always low.

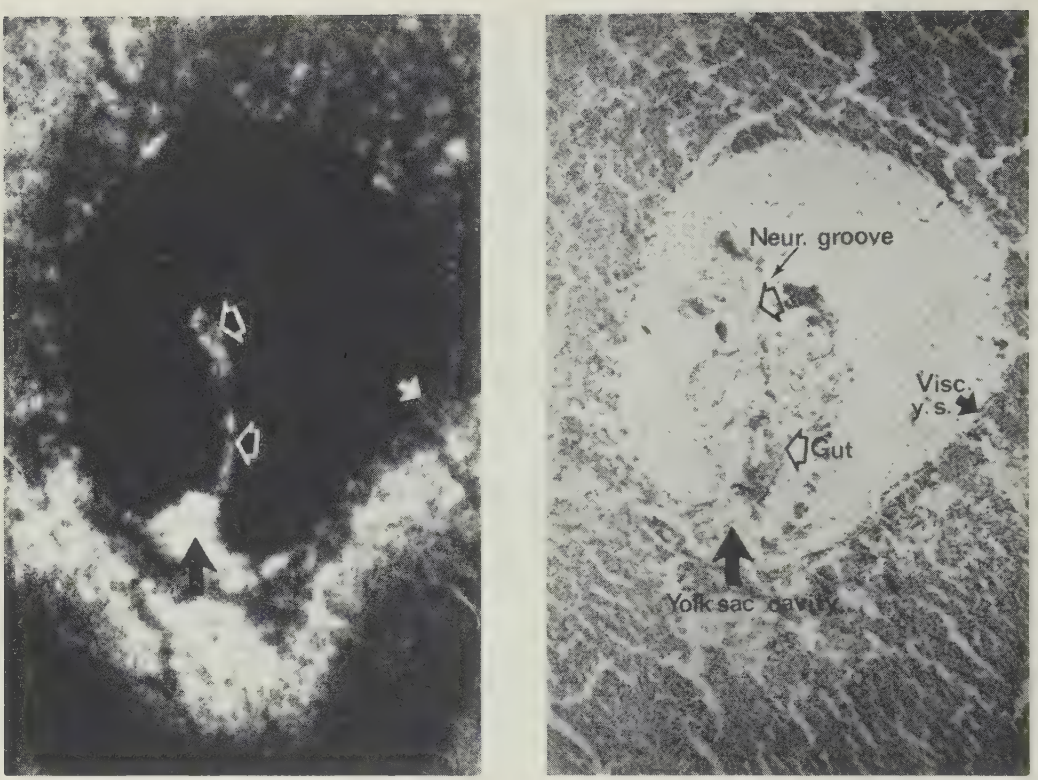


Fig. IV:2. Autoradiogram (left) with the corresponding section (right) showing a mouse embryo and the surrounding decidua. The mother was injected with ^{14}C-2,4,5-T at 8 days 8 hours of gestation and was killed 12 hr later. All embryonic tissues are void of radioactivity except the gut where a high concentration is seen. This radioactivity is continuous with that of the yolk sac cavity, where a high concentration is also seen. No accumulation can be seen in the visceral yolk sac endoderm (compare fig. IV:5) (× 32).

Impulse counting

This experiment was performed to make a quantitative evaluation of the autoradiographically observed increase in the fetal concentration with advancing gestational age. As in chapter III this was done by comparing the ratio of the fetal concentration (per gram tissue) and the maternal serum concentration (per ml) on the different days. The ratio for the maternal liver and the kidney cortex respectively and the maternal serum were also measured. Table IV:2 shows the ratio for the maternal organs. The variations between the different days of gestation are not significant ($p>0.05$).

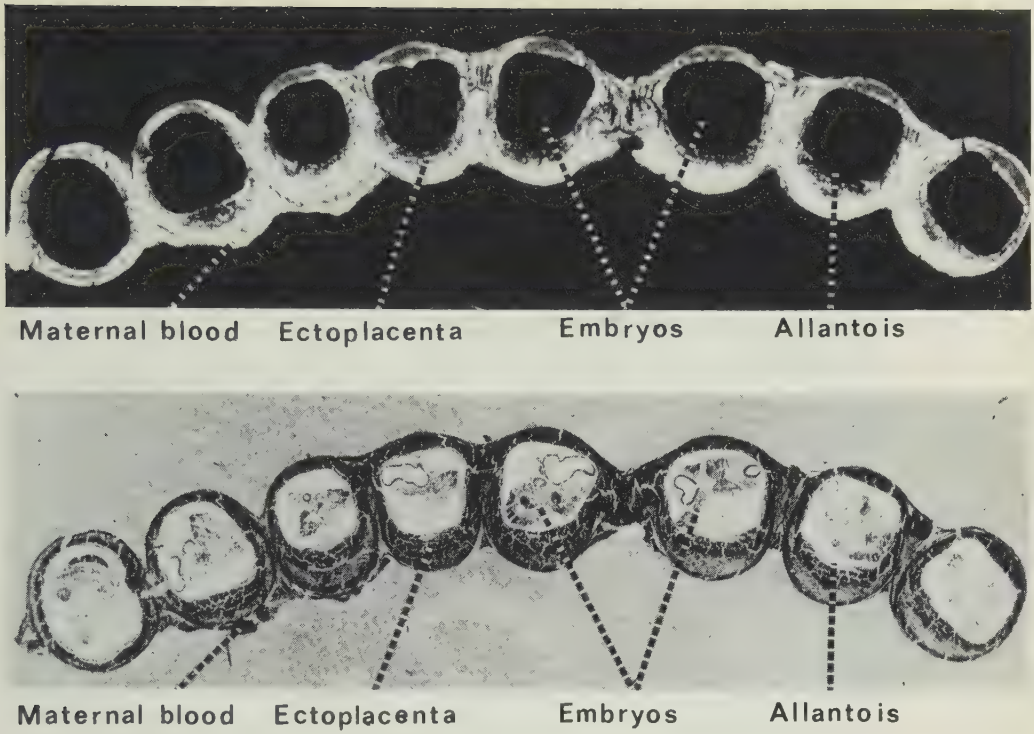


Fig. IV:3. Autoradiogram (upper) and the corresponding section (lower) of a mouse uterus 8 hr after the injection of ^{14}C-2,4,5-T at 10 days 8 hours of gestation. No activity can be seen in the embryos (× 3).

Table IV:3 and fig. IV:6 show the radioactivity distribution ratio between fetuses and maternal serum on the different days of gestation. This ratio increased about 20-fold from day 12 to day 18. If the increase in this ratio is correlated to the fetal growth rate presented in fig. III:7, it is found that during the same period the total amount of 2,4,5-T transported to the fetuses increases about 500-fold.

The variation in the fetal tissue/maternal serum radioactivity distribution ratio between the different days of gestation was highly significant. This was true also between mothers on the same day, on days 16 and 18 due to the small distribution of the values within each mother. The variation between days, however, was of quite another magnitude. The distribution ratio found on day 11 is not presented in the table and figure. The autoradiographic results pointed to a

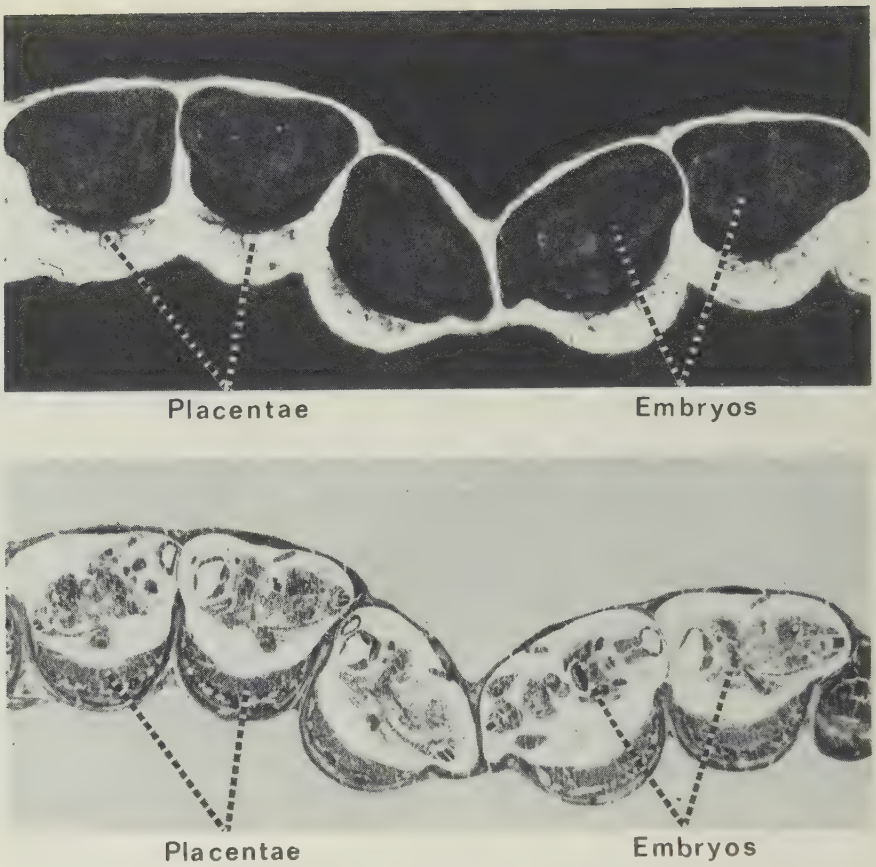


Fig. IV:4. Autoradiogram (upper) and the corresponding section (lower) of a part of a mouse uterus 8 hr after the injection of ^{14}C-2,4,5-T at 12 days 8 hours of gestation. Radioactivity is now visible in the embryos, and it is evenly distributed in all the tissues (× 3).

considerably lower concentration in the fetuses on day 11 than on day 12, while in the impulse counting experiment the mean fetal tissue/maternal serum distribution ratio was higher on day 11 (0.0082) than on day 12 (0.0074). There was also a considerable variation in this ratio of the different embryos on day 11. Several factors may contribute to errors in the quantitative evaluation of the young embryos. The most important of these may be the risk of collecting extraembryonic membranes or traces of maternal tissues, blood or tissue fluid together with the embryos. Taking into consideration the extremely low weight of the embryos, the high concentration of radioactivity in such contaminants would very much affect the results.

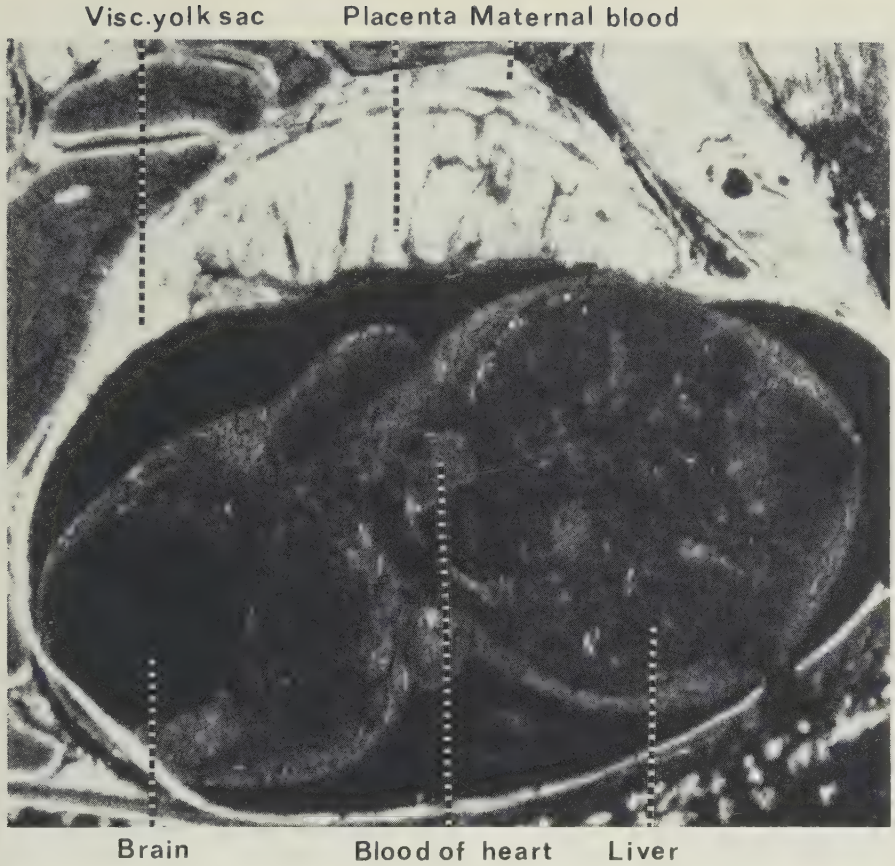


Fig. IV:5. Close up of the fetus shown in fig. IV:1. The fetal concentration is high compared to that shown in figs IV:3 and IV:4. There is a concentration fall from the maternal blood to the placenta and to the fetal blood. An accumulation can be seen in the visceral yolk sac. Note the low concentration in the fetal brain (× 8).

DISCUSSION

Although several reports have appeared concerning the teratogenic effects of 2,4,5-T only that of Neubert & Dillmann (1972) gives more detailed information about sensitivity periods. As in the present study these authors also used mice. They could induce a cleft palate by prolonged treatment (day 6-15). This occurred however also after single higher doses. The period of highest sensitivity in this case

TABLE IV:2. Radioactivity distribution ratio between maternal liver and kidney (per gram) respectively and serum (per ml) 4 hr after the intravenous injection of ^{14}C-2,4,5-T in pregnant mice on different days of gestation. Each value represents the mean ± S.E. with the number of animals in brackets.

Day of gestation	*Liver*	*Kidney (cortex)*
11	0.21 ± 0.01 (4)	1.61 ± 0.07 (4)
12	0.19 ± 0.00 (4)	1.58 ± 0.06 (4)
14	0.23 ± 0.01 (4)	1.92 ± 0.20 (4)
16	0.21 ± 0.01 (2)	1.58 ± 0.06 (2)
18	0.25 ± 0.03 (4)	2.50 ± 0.43 (4)
Significance level (between days)	p>0.05	p>0.05

TABLE IV:3. Radioactivity distribution ratio between homogenized fetuses (per gram) and maternal serum (per ml) 4 hr after the intravenous injection of ^{14}C-2,4,5-T in pregnant mice on different days of gestation. Each value represents the mean ± S.E. with the number of sampling units within brackets. To the left each fetus was used as the sampling unit and to the right the mean of the distribution ratios of all fetuses from each mother was used as the sampling unit. The significance level for the comparison between mothers on the same day of gestation is given for the left column and for the comparison between different days under each column.

Day of gestation	*Each fetus= =one sampling unit*	*p*	*Mean within the mother= =one sampling unit*
12	0.0074 ± 0.0003 (27)	<0.05	0.0072 ± 0.0004 (4)
14	0.027 ± 0.001 (33)	>0.05	0.027 ± 0.001 (4)
16	0.045 ± 0.001 (12)	<0.005	0.045 ± 0.004 (2)
18	0.146 ± 0.004 (28)	<0.001	0.146 ± 0.011 (4)
Significance level (between days)	p<0.001		p<0.001

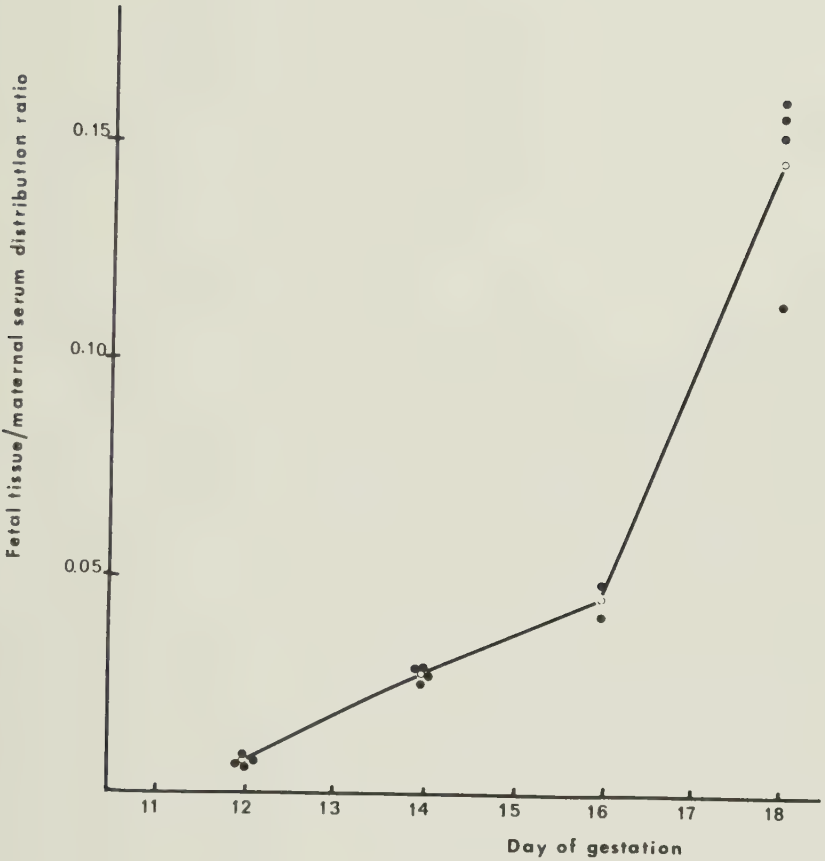


Fig. IV:6. Variations in radioactivity distribution ratio between whole fetuses (per gram tissue) and maternal serum (per ml) as a function of gestational stage. ^{14}C-2,4,5-T was injected into mice 4 hr prior to sampling on the days indicated. The points indicate the mean ratio within each mother and the lines connect the means on the respective days based on each fetus as the sampling unit (table IV:3, left column). There is about a 20-fold increase in the ratio from day 12 to day 18.

was on day 12 and 13, corresponding to day 13 and 14 in the present study. Also Frohberg (1974) found a high malformation rate of 2,4,5-T when administered to mice on day 11-15.

A cleft palate seems to be the most important malformation produced by 2,4,5-T according to all reports found. Also cystic kidneys and minor skeletal changes in addition to growth retardation, delayed ossification and especially late resorption have been found (Courtney & Moore 1971; Roll 1971; Neubert & Dillmann 1972; Båge et al. 1973).

2,4,5-T has a low acute toxicity (LD_{50} 600-900 mg/kg in mice) according to Roll (1971), and therefore a teratogenic effect of 2,4,5-T by a primary damage to the mother may not be probable. As for other substances a teratogenic effect directly on the fetus should first be considered. All the above-mentioned damages and the sensitivity period reported by Neubert & Dillmann (1972) indicate an affect on the fetus primarily in the late organogenetic phase. No serious or frequently occurring damage has been found that is typical for early organogenesis. As nothing points to a long term retention in early pregnancy in any structure of the uterus, placental structures or embryos, such factors cannot explain a delayed onset of damage.

It is tempting to believe that 2,4,5-T acts directly on the fetal tissues. As has been shown in the present investigation, the fetal concentration reaches however an appreciable level only during the late organogenetic stage and therefore affects developmental events only during this period - of which palatal closure is the most sensitive. It may be mentioned that the palatal processes meet mesially first on day 15 (Rugh 1968). There was no specific uptake in the palatal shelves that could explain the specific influence on their development.

If the argumentation above is true there is, also in this case, a good correlation between fetal uptake and damage of the substance in question. It should be considered that the uptake in the embryonic gut early in gestation may also have some influence on the organogenetic processes. An additional possibility, that has been pointed out by Lindquist & Ullberg (1971), is that the yolk sac function may be affected and that this could disturb the fetal nutrition. From day 11 and subsequently there was an increasing uptake in this placental structure.

Another essential question concerns the route of transport of 2,4,5-T to the fetus and the related problem of the rationale for the changes in the fetal concentration with gestational age. As this

problem is essentially the same as that of inorganic mercury, the reader is referred to chapter III (discussion). 2,4,5-T seems to be reversibly bound to the plasma proteins. In the rat about 90 per cent was bound *in vitro* irrespective of the concentration of 2,4,5-T (between 0.17 and 1666 μM) (Piper et al. 1973). It means that there is a part of the plasma content which is ready to diffuse in simple membranes. As 2,4,5-T is a relatively strong acid ($pK_a \sim 3$ according to Erne 1966 a) most of the nonbound part must be in an ionic form at the physiological pH and should not easily pass through the placenta. It has been shown however, in rats of late gestation that anionic drugs pass much more rapidly to the fetus than do cationic ones, despite the fact that they have the same distribution coefficient in a two-phase system of organic solvent and pH 7.4 phosphate buffer (Maickel & Snodgrass 1973). This fact may very well explain the relatively high fetal concentration in late gestation.

It is more difficult to explain the dramatic change in the fetal concentration from day 10 or 11 and up to day 18. One probable explanation is that the fetal membranes including the visceral yolk sac placenta have such a low permeability for 2,4,5-T that this substance does not reach the embryo in autoradiographically detectable amounts early in gestation. With the beginning of rapid development of the chorioallantoic placenta from day 10 or 11 and up to day 18 the anatomical requirements for a transplacental passage are successively fulfilled. It may be of interest to compare 2,4,5-T to another organic acid, viz. salicylic acid (see chapter V). The latter reaches relatively high concentrations in the embryo and fetus at all stages of gestation (which probably means that it must penetrate the yolk sac placenta). Consequently the development of the chorioallantoic placenta only contributes to about a 50 per cent increase in the fetal concentration between day 12 and 18 (if this at all is the cause of the observed increase). Other factors of possible importance for the increased fetal uptake of these two compounds are changes in serum protein binding (Behrman & Battaglia 1967; Curry 1970; Eliason & Posner 1971), serum protein concentration (Kelleher & Villee 1962; Mendenhall 1970), and hydrogen ion concentration both in the maternal and fetal serum with advancing gestational age. These factors can hardly explain the striking change that takes place for 2,4,5-T.

The increase in transfer of 2,4,5-T to the fetus with advancing gestation is so remarkable compared to the other substances used in this study that one wonders if a maturating physiological transport

system is involved. In fact, 2,4-D and 2,4,5-T have been shown to be actively accumulated (transported) by the renal para-aminohippuric acid transporting system (Berndt & Koschier 1973; Hook et al. 1974). Such a system seems however to be lacking in the placenta - at least in sheep (McNay et al. 1969).

As for mercury the most probable explanation for the increasing fetal concentration of 2,4,5-T in late gestation is the successive change in the chorioallantoic blood flow and cellular permeability with a subsequent increase in the passive diffusion of this substance.

The radioactivity recorded in the present investigation most likely represents almost only unchanged 2,4,5-T - as it is very stable in the body (Levey & Lewis 1947; Erne 1966 b). Fang et al. (1973) found only unchanged and unconjugated 2,4,5-T in the blood and liver of rats.

CHAPTER V

SALICYLIC ACID: FREE PASSAGE TO THE CONCEPTUS THROUGHOUT GESTATION

The fact that salicylates may be transferred to the human fetus has been known for about a hundred years. Zweifel (1877) suspected a relationship between salicylate intake by the mother and icterus neonatorum in the newborn child. In 1959 it was found that this drug may produce a variety of severe malformations in the rat (Warkany & Takacs 1959). Later it has been found that this occurs in most animal species (see review by Larsson 1971), including the Rhesus monkey (Wilson 1971). There are indications of teratogenic effects in man, too (Richards 1969). While 2,4,5-T (see chapter IV) produces predominantly cleft palate, especially when administered on day 13 and 14 of pregnancy in the mouse, salicylates cause a variety of malformations and the structure most severely damaged will often be the one in most rapid development at the time of the administration (day 9-11 in the rat) (Kimmel et al. 1971). Organ systems, the primordia of which were not present at the time of treatment, were also involved. In the mouse a variety of malformations were seen (Larsson et al. 1963, 1964; Larsson & Boström 1965; Trasler 1965) among which cleft lip and blood vessel and skeletal anomalies were the most common, but also the palate, the central nervous system, the eyes and extremities were involved. In late gestation fetal hemorrhages and death were produced (Larsson & Eriksson 1966). Salicylates have been found in the embryos or fetuses after administration to the mother of various species in different stages of gestation (Eriksson & Larsson 1971; Kimmel et al. 1971; Boulos et al. 1972; Maickel & Snodgrass 1973; Tjälve et al. 1973). To my knowledge no study has been performed which follows the changes in fetal uptake and distribution of the salicylates as a function of the gestational age. The present autoradiographic and scintillation counting experiments were done to follow the changes in distribution throughout gestation, and to possibly correlate the accumulation in the conceptus with known embryonic and fetal disturbances.

Labelled compound

The radioactive substance, salicylic acid(carboxyl-^{14}C), here called ^{14}C-salicylic acid, was obtained from the Radiochemical Centre, Amersham, England. The spec. act. was 57 mCi/mmol (404 µCi/mg). It was dissolved in physiological saline and analysed for radiochemical purity by thin layer chromatography on silica gel in a solvent system of cyclohexane:chloroform:acetic acid 20:80:10, whereby no impurity was found.

Animals

Mice of the C57BL strain were used. The environmental conditions and mating procedure were the same as those described in chapter I.

Autoradiography

Mice received 10 µCi or about 25 µg ^{14}C-salicylic acid dissolved in 100 µl physiological saline per 20 g body weight intravenously in a tail vein. On day 9, two mice were injected at 8.00 hours and were allowed to live for 4 and 30 hr respectively. At 17.00 hours another animal was injected which was killed 4 hr later. On day 10 two animals were injected, one at 8.00 hours and the other at 16.00 hours, and both were killed 4 hr after injection. On each of the days 11, 12, 13, 14, 15, 16 and 17 at 8.00 hours one animal was injected and killed 4 hr later. The animals were killed by prolonged ether anesthesia. In the animals pregnant up to day 11, the abdomens were opened and the uteri removed and mounted. The mice on day 12-17 of gestation were mounted intact. Thereafter the uterine tissues or the whole animals were sectioned and autoradiography was performed as described in chapter I. The exposure time ranged from 3 to 6 months.

Liquid scintillation counting

Four mice on day 12 and three on day 18 of gestation were injected intravenously in a tail vein, each animal receiving 0.25 µCi ^{14}C-salicylic acid dissolved in 2.5 µl physiological saline per gram body weight. This corresponded to about 0.62 µg/g body weight. Four hr after injection the animals were killed and each embryo, pieces of the maternal liver, renal cortex and also serum were collected for liquid scintillation counting as described in chapter IV.

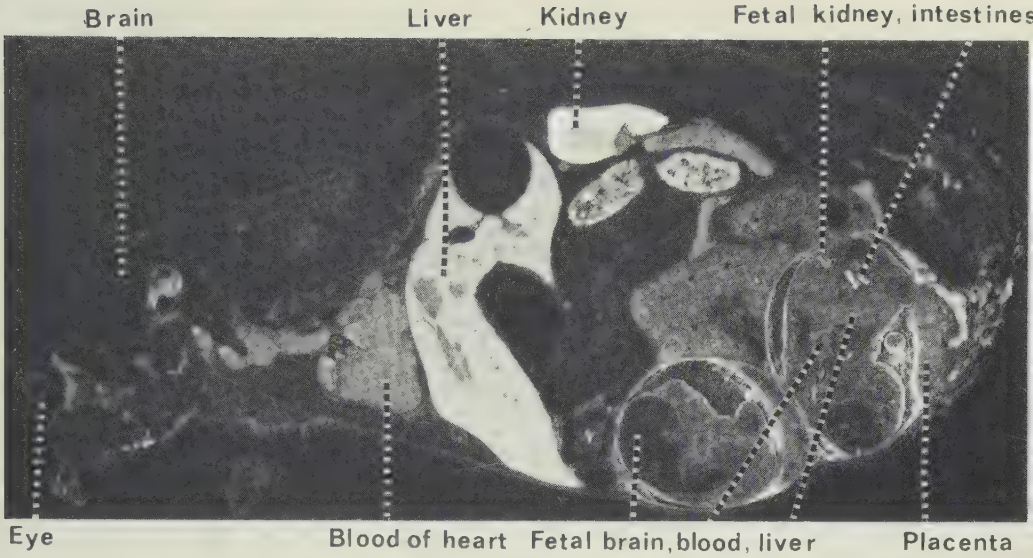


Fig. V:1. Whole body autoradiogram of a mouse on day 17 of gestation, 4 hr after the injection of ^{14}C-salicylic acid. The highest accumulation is seen in the maternal kidney. The liver has only a slightly higher concentration than the blood. From the maternal blood to the placenta and then to the fetal blood the concentration is successively decreasing. Within the fetus the distribution pattern is more even than in the mother. However the brain has a low concentration (as is also seen in the mother), while there is an accumulation in the intestines and kidney. The fluids surrounding the fetus have a rather high concentration.

RESULTS

Autoradiography

The whole body autoradiographic distribution picture of the adult animal (fig. V:1) was found to correspond to that described by Tjälve et al. (1973). The highest accumulation was seen in certain tubuli of the renal cortex. There was also a high concentration of radioactivity in the renal pelvis and in the urinary bladder which shows that this is the principal way of excretion. The liver had a concentration only slightly exceeding that of the blood, and the gall bladder content showed variable concentrations most often slightly higher than that of the liver. All other tissues had a concentration below that of the blood.

Embryo, fetus. From the earliest gestational age studied in this investigation the embryonic concentration was comparatively high 4 hr after injection, but at 30 hr most of the radioactivity had disappeared from the embryos. At this time interval traces of radioactivity were found in the maternal liver and kidney. The concentration in the ectoderm and the neuroepithelium (and later in the brain) was always among the highest in the embryo in the earlier stages (fig. V: 2-5) and sometimes comparable to that of the maternal liver up to day 13. Also the endoderm showed a comparatively high accumulation at these stages. In other "dense" areas, i.e. with high cellular proliferation, a variably high uptake was seen. Undifferentiated mesenchymal tissues had a low uptake at these earlier stages.

From day 14 and subsequently, the higher uptake in the brain tissues was no longer observed (fig. V:6), and the distribution pattern became in fact more even than previously. Later a more differentiated pattern again appeared (fig. V:1). There was a marked uptake in the ribs, long bones and cartilage of the conchae on day 16 and 17. On day 17 a fetal blood-brain barrier was obvious, and there was a higher concentration in the fetal intestinal contents. This could be due to a biliary or intestinal excretion, but it might also depend on the fetal swallowing of amniotic fluid which contained a great amount of radioactivity. A spotty renal accumulation was also observed and a high concentration in the fetal urinary bladder.

Placental structures. The visceral yolk sac did not accumulate the radioactivity at any gestational age. In the yolk sac cavity usually a concentration at the level of the blood was seen in the earlier stages. In the uterine fluid of later gestation a high concentration was variably seen surrounding the visceral yolk sac placenta. The chorioallantoic placenta always showed a lower concentration than the maternal blood.

From day 12 and up to day 17 a steadily increasing concentration was seen in the amniotic fluid and the exocoelom (fig. V:1 and 6). At the last days of gestation this activity may partly be due to a fetal

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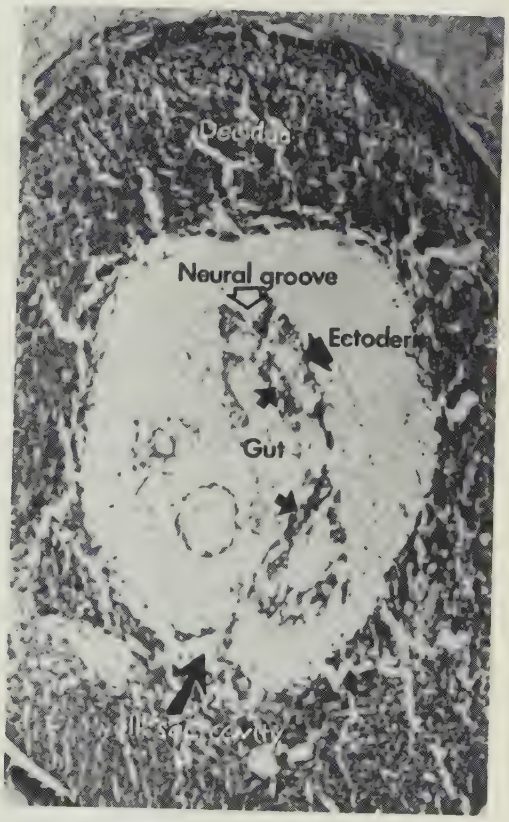
Figs V:2 (upper) and V:3 (lower) (on the opposite page). Autoradiograms (left) and the corresponding sections (right) of embryos and the surrounding uteri of mice injected at 8 days 8 hours (V:2) and 8 days 17 hours (V:3) respectively and killed 4 hr later. The embryonic tissues have an average concentration comparable to that of the uterine tissues. An especially high concentration can be seen in the ectoderm, neuroepithelium, gut and in the yolk sac cavity (V:3) (× 32 resp. × 24).

Embr. ectoderm
Gut


Neural groove
Gut

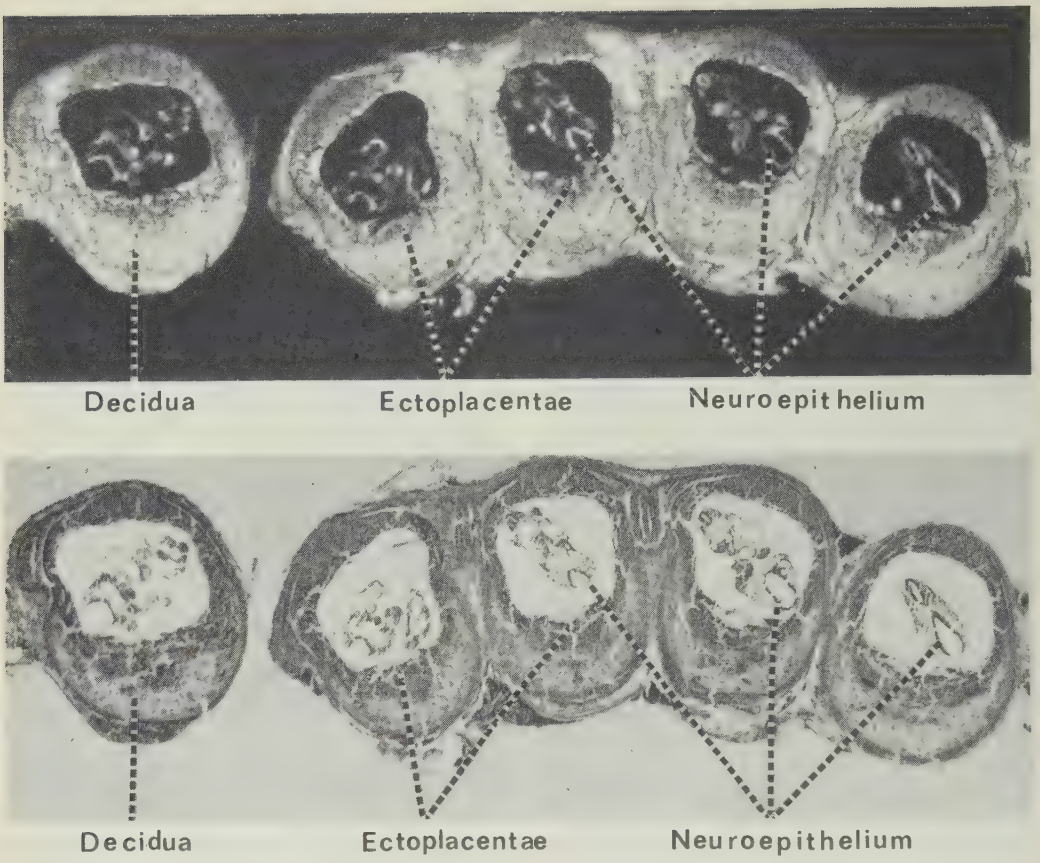


Fig. V:4. Autoradiogram (upper) and the corresponding section (lower) of a part of a mouse uterus showing 5 different embryos. The mother was killed 4 hr after the injection of ^{14}C-salicylic acid at 9 days 16 hours of gestation. The highest concentration in the embryos is found in the neuroepithelium. A variably high uptake also can be seen in other "dense" areas, having a high cellular proliferation (× 6).

urinary contamination, while at the earlier stages it probably had diffused there via the fetal membranes. This radioactivity was often seen to collect on the fetal skin and the amniotic and yolk sac membranes which had probably occurred during the drying of the sections.

Impulse counting

Even if the autoradiographic distribution picture showed a relatively high embryonic-fetal concentration throughout gestation, it was considered of interest to check whether there was a measurable change in the fetal tissue/maternal serum distribution ratio during

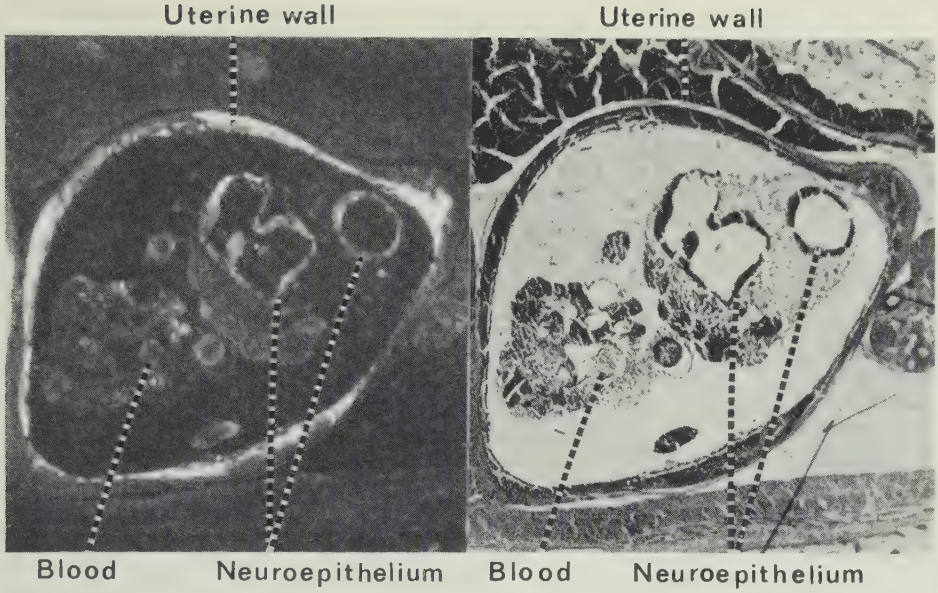


Fig. V:5. Detail of whole body autoradiogram (left) and the corresponding section (right) showing an embryo, the uterine wall and the surrounding maternal tissues. The mouse was injected with ^{14}C-salicylic acid at 11 days 8 hours and killed 4 hr later. The highest concentration within the embryo is found in the neuroepithelium. The concentration in the surrounding maternal tissues is lower. The embryonic blood concentration seems to be an average of the overall fetal concentration (× 8).

the last week of pregnancy - the period when an impulse counting experiment could be expected to give reliable results.

As can be seen in table V:1 there were variations in the fetal tissue/maternal serum ratio between the different mothers on the same day of gestation. The variations between the different fetuses from the same mother were not very great, however. This means that there was a statistically significant variation between the mothers. There was also a statistically significant increase in the mean fetal tissue/maternal serum ratio between day 12 and day 18. This was highly significant if the single fetus was used as the sampling unit, but significant also when the mean fetal tissue/maternal serum ratio within each mother was used as the sampling unit.

There was a statistically significant increase in the maternal liver/serum distribution ratio from day 12 to day 18, and simultane-

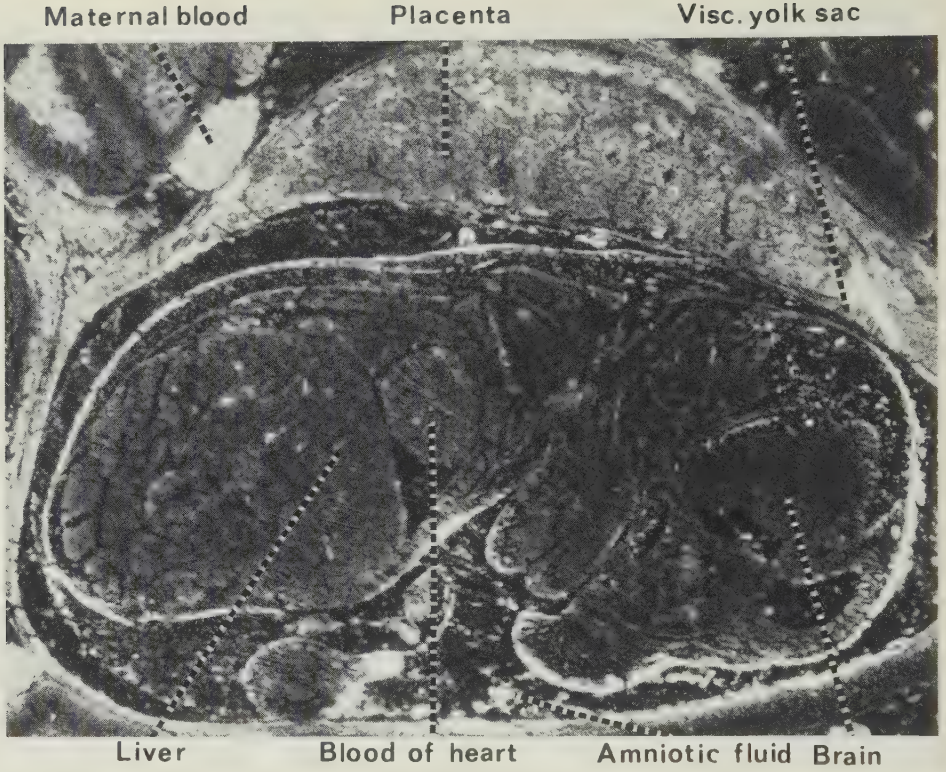


Fig. V:6. Detail of a whole body autoradiogram showing a mouse fetus and the corresponding placenta. The mother was killed 4 hr after the injection of ^{14}C-salicylic acid at day 15 of gestation. The radioactivity is evenly distributed within the fetus (compare fig. V:1 and V:2-5). The concentration is higher in the maternal than in the fetal blood. In the amniotic fluid there is a rather high concentration and it is probable that the radioactivity seen on the skin of the embryo has gathered there during the drying of the section (× 10).

ously there was a decrease in the maternal kidney/serum ratio. The reasons for this change are not understood, but it may be explained by alterations in the excretory function of the two organs with advancing gestation.

TABLE V:1. Radioactivity distribution ratio between tissues (maternal liver, kidney, collected fetal tissues) (per gram) and maternal serum (per ml). It is 4 hr after the intravenous injection of ^{14}C-salicylic acid in pregnant mice on day 12 and 18. Mean ± S.E.

DAY 12

	Mother 1	2	3	4	*Mean ± S.E.*
Liver	0.68	0.78	0.87	0.77	0.78 ± 0.04
Kidney	3.47	3.33	4.08	3.00	3.47 ± 0.23
Fetus 1	0.18	0.24	0.21	0.17	
2	0.16	0.25	0.20	0.16	
3	0.16	0.24	0.22		
4		0.23	0.20		0.20 ± 0.08
5			0.22		
6			0.21		
7			0.20		
Mean ± S.E.	0.17 ±0.01	0.24 ±0.00	0.21 ±0.00	0.17 ±0.01	

$p<0.001$ (between mothers; sampling unit=each fetus)

DAY 18

	Mother 1	2	3	*Mean ± S.E.*
Liver	1.14	0.90	0.99	1.01 ± 0.07
Kidney	2.45	2.22	3.00	2.56 ± 0.23
Fetus 1	0.29	0.28	0.30	
2	0.33	0.24	0.27	
3	0.29	0.30	0.26	
4	0.32	0.27	0.31	0.29 ± 0.05
5	0.33	0.27	0.29	
6	0.29	0.26	0.24	
7	0.31	0.30	0.27	
8	0.33		0.30	
9	0.33			
Mean ± S.E.	0.31 ±0.01	0.27 ±0.01	0.28 ±0.01	

$p<0.005$ (between mothers; sampling unit=each fetus)

Significance level between day 12 and day 18

Maternal liver	$p<0.05$
" kidney	$p<0.05$
Fetus (sampling unit=each fetus)	$p<0.001$
(sampling unit=mean fetal conc. within mother)	$p<0.05$

When compared to the finding of inhibited transport of 2,4,5-T in early gestation (see chapter IV), the present results showing that salicylic acid passes to the fetus during all periods of development studied are of interest. Salicylates are also taken up in both the unimplanted and the implanted blastocysts (Lutwak-Mann 1962). It seems therefore that all the morphological changes in the membranes and the placental structures that occur throughout gestation do not dramatically change the transport of salicylic acid to the conceptus - as was the case with 2,4,5-T.

Sturman & Smith (1967) and Sturman et al. (1968) found that while the salicylates showed a high plasma protein binding in man, it was below 10 per cent at all serum concentrations studied in e.g. the mouse and the rat. This may be one factor that favours membrane transport of salicylic acid. Even if it must be predominantly ionized at the physiological pH ($pK_a \sim 3$ according to Weiner et al. 1959), non-ionic diffusion probably explains the relatively high embryonic-fetal concentrations throughout pregnancy.

In early pregnancy, before chorioallantoic circulation begins, the passage to the embryo must take place via the visceral yolk sac endoderm - by entering the vitelline vessels and following the circulation to the embryo. It can also occur by passing *through* the visceral yolk sac wall into the exocoelomic cavity and further through the amnion and amniotic cavity. The high activity in these cavities and membranes in late pregnancy speaks in favour of the latter hypothesis, and this must be the case before the vitelline circulation begins (on day 9). On the other hand, there was little activity present in the fluids surrounding the embryo in early gestation. The two routes may probably be followed synchronously.

From day 12 to day 18 the radioactivity distribution ratio between homogenized fetal tissues and the maternal serum increased almost 50 per cent which is a low increase compared to 2,4,5-T (showing a 20-fold increase). As the distribution pattern of salicylic acid within the fetus markedly changed during the same period, it is not possible to confirm whether the observed increase reflects a similar increase in the fetal serum/maternal serum ratio. The fetal blood concentration seemed to be an average of all the fetal tissues throughout this period (as judged from the autoradiograms). If this is

true it is probable that the changes occurring in the chorioallantoic placenta during the same period (described in chapter III) were responsible for the increased transfer of salicylic acid. It seems unlikely that the increase in the fetal dry weight percentage and protein concentration (shown in chapter III) were responsible because the distribution pattern in the late fetus showed a relatively high concentration in the body fluid and in the excretory organs, while in the embryo and early fetus the uptake was seen in the rapidly developing structures. Another possible explanation for the increased ratio is a change in the maternal and/or fetal serum (e.g. protein or hydrogen ion concentration; see chapter IV, discussion) and this must be considered.

As was discussed in the introduction, fetal damage can be caused by salicylates during most stages of development. This corresponds well with the free passage of salicylic acid to the embryo-fetus. It is more questionable to correlate a moderate uptake in certain tissues or organs to a specific damage. The more sensitive structures will probably reach concentrations high enough to be damaged. The high uptake in the neuroepithelium in early pregnancy may be correlated to exencephaly, hydrocephaly, spina bifida, and the eye defects found in rats (Warkany & Takacs 1959) and mice (Trasler 1965). The uptake in the skeletal structures during the last days of gestation may be related to the defects in these parts reported by Larsson et al. (1964), even if they were most often produced in early pregnancy.

The mechanism of teratogenic action of the salicylates may still be considered unknown. Interference in the mucopolysaccharide synthesis has been shown to occur (Larsson & Boström 1965), and the salicylates are known to inhibit the RNA polymerase activity in rat fetuses (Janakidevi & Smith 1970). Human embryonic cell culture growth has also been depressed (Paine & Nagington 1971). Attention has been focused on salicylate inhibition of prostaglandin synthesis during the last years (Vane 1971; Smith & Willis 1971). This effect of the salicylates may have implications in regard to the fetal damage.

The placental barrier is often compared to the blood-brain barrier. Cationic compounds seem to be blocked about equally well in both these barriers (Allgén et al. 1960; Hansson & Schmiterlöw 1961; Maickel & Snodgrass 1973). Anionic substances, on the other hand, seem to pass much more readily to the fetus than to the brain (Maickel & Snodgrass 1973). When one considers the late stages of gestation, salicylic acid and 2,4,5-T agree well with this concept. A possible

explanation of the differences between anions and cations may be that the anions more or less pass to the brain as well but are removed by the organic acid transporting system of the choroid plexus. This latter structure has been shown to accumulate salicylic acid (Lorenzo & Spector 1973). Such a transport system has been reported as not present in the placenta of sheep (McNay et al. 1969). There may, of course, also be a real difference in these two "barriers". Factors such as the hydrogen ion and protein concentration of the pertinent compartments may also differ, influencing the rate of diffusion.

In the mouse, besides salicylic acid only some salicyl glucuronides were found in all the tissues except the brain after injection of salicylic acid (Sturman et al. 1968). In the rat, which excreted salicyl glucuronides in the urine, only salicylic acid was found in the serum and the embryos after injection of acetylsalicylic acid (Kimmel et al. 1971). It is probable that also in the mouse mainly salicylic acid and not its metabolites pass to the embryo.

Salicylates are known to pass to the human fetus in late gestation (Zweifel 1877; Jackson 1948). Nothing is known about its passage in the early organogenetic period. The results of the present investigation, showing the ease with which salicylic acid penetrates all barriers of the mouse conceptus, makes probable a similar ability of salicylic acid to penetrate in the human conceptus.

The observation of Kimmel et al. (1971) that the simultaneous administration of benzoic acid, which is structurally related to salicylic acid, increases the embryonic concentration of salicylic acid should be noted. Benzoic acid is widely used as a food preservative. This or other related compounds may be ingested in too high concentrations along with salicylates by pregnant women, which may be a risk for the fetus.

CHAPTER VI

ZINC: ACCUMULATION IN RAPIDLY GROWING EMBRYONIC STRUCTURES

In this monograph the essential metal zinc justifies its place for at least two reasons: firstly, a zinc deficiency invokes malformations in experimental animals (Blamberg et al. 1960; Hurley & Swenerton 1966), and secondly, zinc can prevent the malformations produced by cadmium (Ferm & Carpenter 1967), which is dealt with in chapter I. These two aspects indicate that zinc is of fundamental importance in embryonic-fetal development. Zinc is a frequently occurring metal in living organisms and their different enzyme systems (Schroeder et al. 1967). Among the effects produced by a zinc deficiency, the one resulting from an impaired DNA synthesis (Lieberman & Ove 1962; Lieberman et al. 1963), is probably the most interesting when one considers embryonic development. Swenerton et al. (1969) showed that a zinc deficiency reduced thymidine incorporation in the rat embryos.

This autoradiographic study of the zinc distribution in mice and hamsters was undertaken to obtain knowledge concerning the embryonic-fetal uptake of a physiologically interesting substance for the above mentioned reasons. In addition, the possible effects of simultaneously administered cadmium in teratogenic doses on the distribution of ^{65}Zn in early hamster embryos were studied.

MATERIALS AND METHODS

Isotope and chemical

Carrier free $^{65}ZnCl_2$, spec. act. 262 μCi/μg, dissolved in 0.1 M-HCl was obtained from the Radiochemical Centre, Amersham, England. $CdCl_2$ (anhydrous powder, 99.7 per cent pure) was obtained from J.T. Baker, Chemical Co., Phillipsburg, N.J., U.S.A.

Animals

Golden hamsters and mice (C57BL) were used and breeding and environmental conditions were the same as those described earlier. The hamsters, weighing about 110 g, received 50 µCi or 0.19 µg (i.e. about 0.0017 µg/g body weight) dissolved in 100 µl 0.043 M-HCl. The mice, weighing about 25 g, received 20 µCi or 0.076 µg (i.e. about 0.031 µg/g body weight) dissolved in 40 µl. The animals were injected intravenously as described earlier. The hamsters were injected on the gestational day 8: at 10.00 and 15.00 hours and killed 24 hr later; at 16.00 hours and killed 1 hr later; at 22.00 hours and killed 12 hr later. On day 9 two hamsters were injected at 8.00 hours and were killed 4 and 24 hr later, respectively.

Three hamsters on day 8 of gestation received at 16.00 hours 2 mg Cd^{2+}/kg body weight as $CdCl_2$ dissolved in 100 µl distilled water intravenously in a jugular vein, and immediately thereafter $^{65}ZnCl_2$ as described above. They were allowed to live for 1, 4 and 24 hr respectively.

Two nonpregnant mice received $^{65}ZnCl_2$ intravenously. Three days later they were mated and then killed on day 10 and 18 of gestation respectively, which was 13 and 21 days after the injection. Three mice on day 8 of gestation were injected at 10.00, 16.00 and 20.00 hours and were killed 24, 24 and 12 hr later respectively. Two mice were injected on day 9 at 8.00 hours and were killed 4 and 24 hr later respectively: one at 12.00 hours and killed 4 hr later; one at 17.00 hours and killed 16 hr later. Finally one mouse on day 15 was injected and killed 24 hr later. In all the animals the uteri were removed and mounted as described before, except in the two mice injected before pregnancy and the one injected on day 15 of gestation which were mounted intact. Autoradiography was performed as described earlier. The exposure time ranged from 8 to 200 days.

RESULTS

The whole body autoradiographic distribution picture of the mother changed considerably during the long observational period used in this study. Twenty-four hours after the injection (fig. VI:1) there

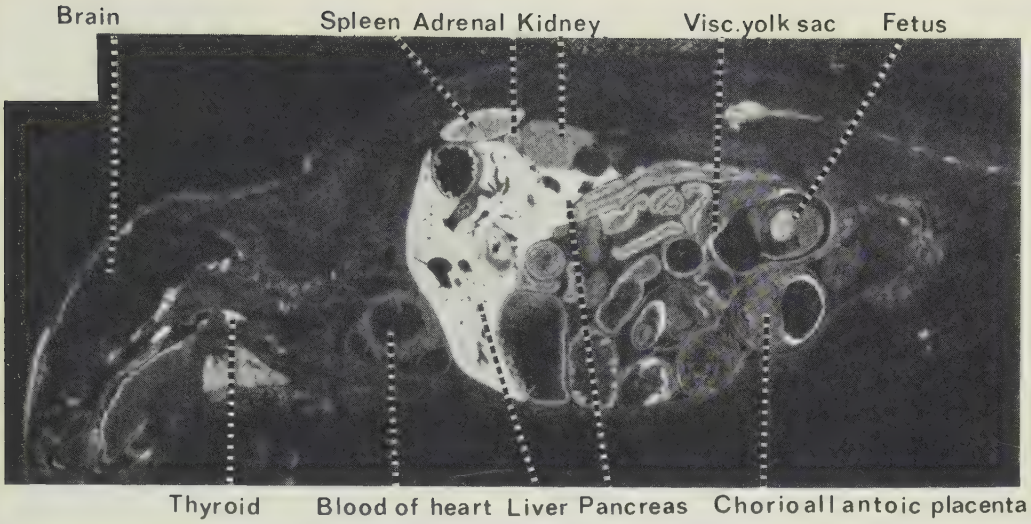


Fig. VI:1. Whole body autoradiogram of a mouse 24 hr after the injection of $^{65}ZnCl_2$ on the 15th day of gestation. The radioactivity has almost disappeared from the blood and accumulated in the different organs. The highest concentration can be found in the liver and pancreas, and in other parenchymatous and endocrine organs. The visceral yolk sac has a high concentration. The average fetal concentration is about equal to that of the mother.

was a rather high uptake in the parenchymatous organs (liver, pancreas, kidneys, and spleen) and also in the bone marrow and in the cutaneous as well as in the mucous epithelia of the alimentary tract. The ovaries showed a moderate accumulation, which was somewhat higher in the follicles than in the corpora lutea. There was even a higher uptake in the epiphyseal lines. At 13 days and even more at 21 days after the injection, the distribution pattern was more uniform except for the calcified structures of the skeleton where a very high concentration was seen (fig. VI:5).

Embryo, fetus

Irrespective of the stage of pregnancy, the time after injection, and whether the dams were injected before or after mating - there was a comparatively high concentration in the embryonic and fetal tissues. However, the concentrations in the early and late pregnancy fetuses were not directly comparable due to the change in the distribution pattern with developmental stage. No differences were observed in the distribution pattern of the hamster and the mouse

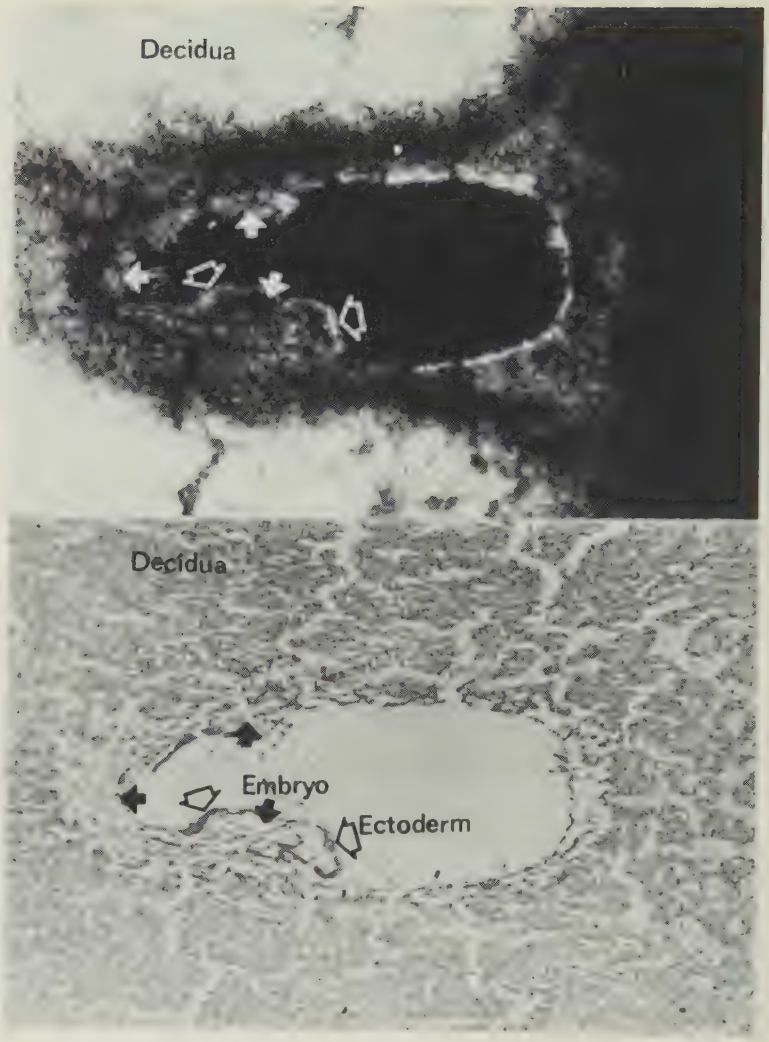


Fig. VI:2. Autoradiogram (upper) and the corresponding section (lower) showing a hamster embryo and the surrounding membranes and decidua. It is 1 hr after the injection of $^{65}ZnCl_2$ into the mother at 7 days 20 hours of gestation. There is a relatively high uptake in the embryo (thick arrows), especially in the ectoderm (empty arrows), and in the antimesometrial part of the decidua (× 28).

embryos.

The highest uptake was seen in the ectodermal layer in early gestation, even as early as 1 hr after the injection on day 8 in the hamster (fig. VI:2). Later, when the organogenesis had proceeded, the highest activity was found in the neuroepithelium, ectoderm, endoderm (gut and the tissues surrounding it - probably the primordia of the organs derived from the endoderm), somites, heart and blood vessel primordia, but less in the loose mesenchyme (fig. VI:3).

In late gestation (24 hours after the injection on day 15, fig. VI:4) the distribution pattern was different. The liver now showed the highest concentration. Some layers of the developing brain showed a higher activity than others. The skeleton also showed a rather high uptake. The fetal blood level was well above the maternal. In the fetuses of the dam injected 3 days prior to mating and killed at day 18 of gestation (fig. VI:5), the liver and the calcified structures dominated the fetal distribution picture. Otherwise the distribution was very even - as in the mother. This included the brain, unlike the situation on day 15.

In the embryos of the hamsters injected with a teratogenic dose of unlabelled cadmium ($CdCl_2$) prior to the administration of the radioactive zinc - no quantitative or qualitative difference in the distribution pattern could be determined when they were compared to those injected with only $^{65}ZnCl_2$. This naturally does not exclude the possibility of a difference which could not be revealed with this method (e.g. on the subcellular level).

Placental structures

There was a pattern of zinc uptake especially in the antimesometrial decidua during early gestation (fig. VI:2) similar to that described for cadmium (chapter I). There was only a weak uptake in the visceral yolk sac at early gestation (fig. VI:3), but the uptake was rather high in the developing chorioallantoic placenta. On day 15 of gestation in the mouse the visceral yolk sac showed a prominent accumulation (fig. VI:1 and 4), while it was lower in the chorioallantoic placenta. Still the chorioallantoic placenta had a concentration higher than that of the maternal blood. At this stage the decidua basalis had a concentration higher than that of the placenta. It is interesting to note that in the 18th day pregnant mouse injected prior to mating there was no accumulation in the visceral yolk sac (fig. VI:5). In the decidua of this animal the concentration was very high.

The present study showed that zinc was taken up in the fetal organs in comparatively high concentrations at all stages of development. It was thus probably transported both through the visceral yolk sac and the chorioallantoic placenta. The embryonic-fetal uptake was high irrespective of whether the radioactive zinc was administered during pregnancy or if it was stored in the maternal body before pregnancy. In the early gestational stages it was concentrated in the most rapidly developing tissues of the embryo (e.g. neuroepithelium, endodermal tissues, somites). This distribution picture may be an expression of the requirements for zinc in enzymes taking part in DNA synthesis (Lieberman & Ove 1962; Lieberman et al. 1963; Fujioka & Lieberman 1964; Slater et al. 1971). It has in fact been shown that a zinc deficiency decreases the thymidine incorporation in the embryonic tissues (Swenerton et al. 1969).

In late pregnancy probably other enzyme systems requiring zinc are developed, which may be the reason why the zinc pattern at this stage was not identical with a thymidine pattern (unpublished observation). In addition, the relatively high liver concentration may be ascribed to a storage function, and in the bone structures zinc probably takes part in the mineralization process.

There are many earlier reports on the placental transfer of zinc. Feaster et al. (1955) found a higher concentration in the 11th day rat embryo than in the full term fetus. Terry et al. (1960) found however an increasing concentration in the rabbit fetus during the last days of gestation - due to liver accumulation. In man the zinc concentrations in the fetal serum were found to be higher than the maternal concentrations (Vikbladh 1951; Berfenstam 1952; Widdowson 1970; Henkin et al. 1971) but equal to the serum levels of normal

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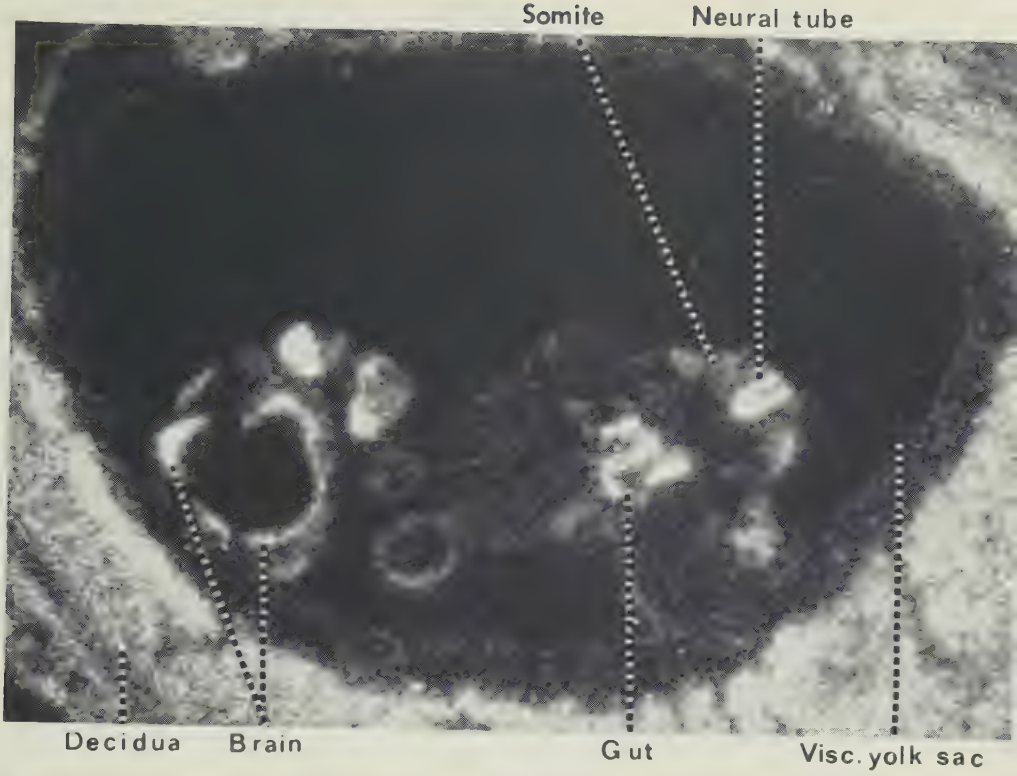
Fig. VI:3 (on the opposite page). Autoradiogram (upper) and the corresponding section (lower) of a hamster embryo and the surrounding structures. It is 24 hr after the injection of $^{65}ZnCl_2$ into the mother at 7 days 19 hours of gestation. The highest concentrations are seen in the neuroepithelium (brain and neural tube), the gut (and close to it what seems to be tissues developed from the endoderm), and some unidentified structure close to the brain (probably neuroepithelium). A rather high concentration is seen in the somites. In the visceral yolk sac there is a low concentration, but it is high in the decidua (× 34).


Somite
Neural tube
Decidua
Brain
Gut
Visc. yolk sac

Somite
Neural tube
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Brain
Gut
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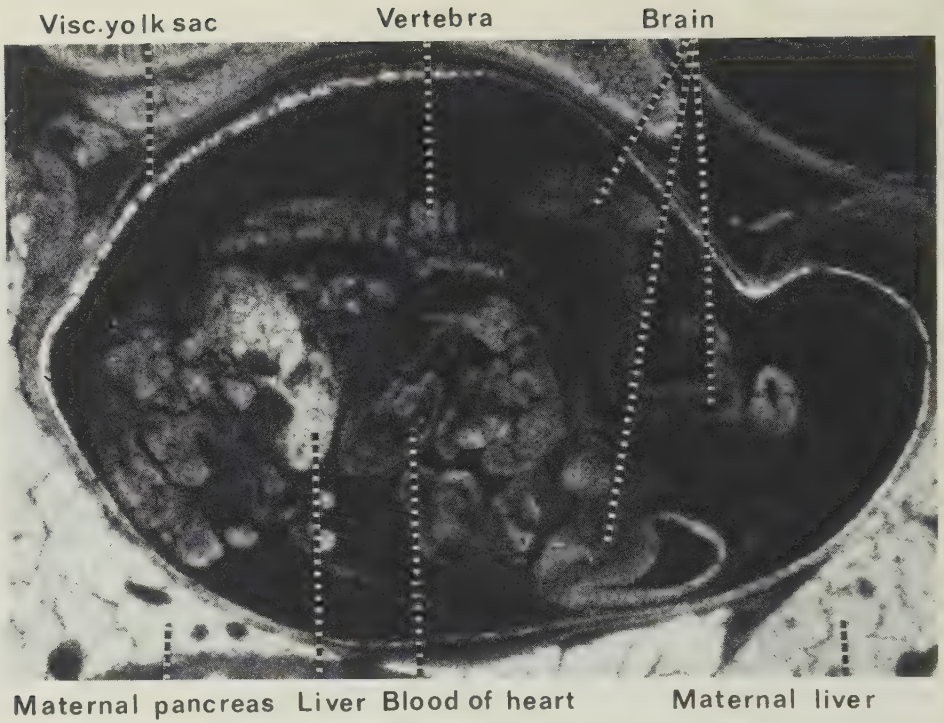


Fig. VI:4. Detail of whole body autoradiogram showing a mouse fetus of the same mother as that shown in fig. VI:1. At this stage there is a relatively high concentration only in some layers of the developing brain. The highest concentration is found in the liver (× 11).

nonpregnant adults (Widdowson 1970; Henkin et al. 1971; Kurz et al. 1973). Immature fetuses had higher levels than the full term fetuses (Widdowson 1970). Autoradiographic studies of late term rat and mouse fetuses have revealed bone, kidney, and liver localizations (Kinnamon 1963; Bergman & Söremark 1968). Ferm & Hanlon (1974) found zinc to pass to the hamster embryo on day 8-9 of gestation. To my knowledge the specific localization of zinc in rapidly developing areas of the embryo-fetus has not been reported earlier, nor the interesting change in its distribution pattern from early to late gestation.

The zinc of human serum is to 30-40 per cent unexchangeably bound to an α_2-macroglobulin fraction and the remaining part is exchangeably bound to albumin (Parisi & Vallee 1970). It seems probable that the latter fraction is that which is most readily transferred to the embryo. Henkin et al. (1971) found part of the serum zinc to be ultrafiltrable and proposed a passive transfer of this fraction. The

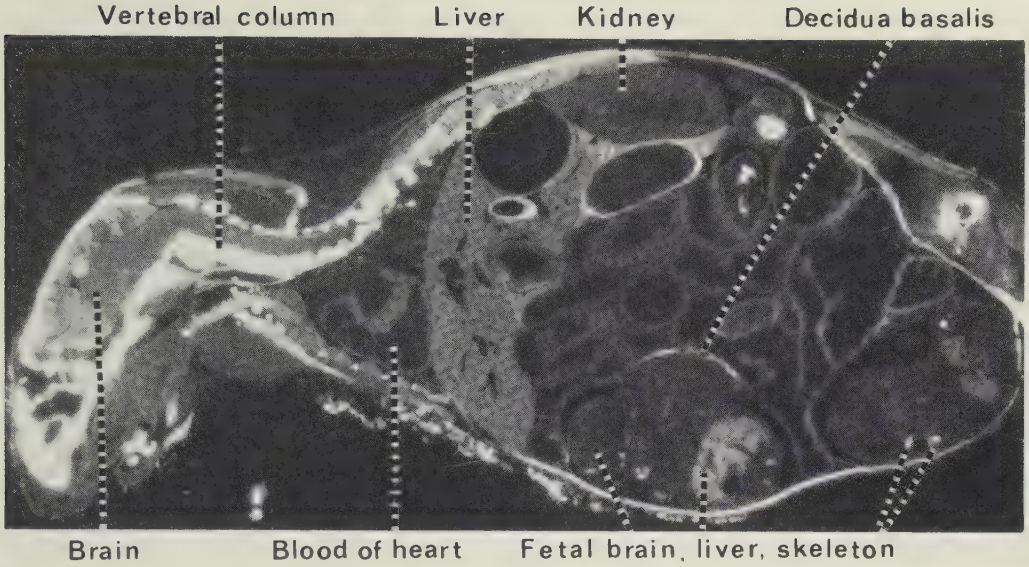


Fig. VI:5. Whole body autoradiogram showing the distribution picture of $^{65}ZnCl_2$ in a mouse on the 18th day of gestation. It was injected 21 days earlier, 3 days before mating. With the exception of the maternal skeleton which has a very high concentration, the fetal tissues have a concentration close to the maternal. The fetal liver has a somewhat higher concentration than the maternal one while the brain has a much lower concentration than the maternal brain. There is an accumulation in the decidua basalis, while the concentration in the visceral yolk sac is low.

high ratio of zinc transfer to the embryo compared to that of cadmium and mercury found in the present study suggests a more active mechanism of transfer.

Several experiments on the teratogenic effects of zinc deficiency in rodents have shown that this metal is essential for embryonic development. Thus only a short period of a restricted diet is required for the induction of severe malformations. Most organs usually investigated seem to be damaged, the type of damage depending on the time of zinc depletion (Blamberg et al. 1960; Hurley & Swenerton 1966; Hurley et al. 1971; Warkany & Petering 1972, 1973). The comparatively high concentration in the neuroepithelium indicates the high need for zinc in this structure. This may very well explain the severe central nervous system damage produced by its deficiency (Warkany & Petering 1972, 1973).

It has been claimed that the relative ease with which zinc deprivation damages the embryo must be due to difficulties in mobilizing

zinc from the maternal depots (Hurley et al. 1971; Warkany & Petering 1973). The fact that the fetuses reached the same concentration as the mother even when the isotope was injected before pregnancy shows, however, that at least under normal conditions there is a turnover of the maternal depots, and that the zinc released from these depots is shared by the fetuses. It is most probable that this happens also in a deficiency state, but that the depletion is more dangerous to the embryo than to the mother. The behaviour of zinc is in this respect quite different from that of the nonessential cadmium, which was not seen in detectable amounts in either the fetus or the placental structures when administered before pregnancy (see chapter I). The turnover of cadmium is low compared to that of zinc (Cotzias et al. 1961; see also Webb 1972). It is interesting to note that when the zinc was already stored in the mother before pregnancy, there was no accumulation in the late stage visceral yolk sac endoderm, but it was high 24 hr after the injection in late stage pregnancy. There may be two reasons for this difference: there is either a rapid transport from the uterus to the yolk sac, but only a slow one from the yolk sac to the fetus; or the zinc reaches the visceral yolk sac in a less physiological form at instantaneous administration but bound to specific carrier proteins at a slow, continuous supply to the placental structure. Zinc bound to specific carriers might be more readily transported by the visceral yolk sac and therefore not accumulated in this structure. In that case the visceral yolk sac accumulation after instantaneous administration might be considered an artifact.

The most interesting finding that zinc prevents the teratogenic effects of cadmium (and also interferes with mercury teratogenesis) (Ferm & Carpenter 1967; Gale 1973), makes it probable that cadmium causes its damage by interfering with the embryonic zinc metabolism. Such an interference has been reported for a number of biological processes in adult animals (reviewed by Friberg et al. 1974). It perhaps should not be surprising that it was impossible to determine a major change in the zinc distribution by the simultaneous administration of teratogenic doses of cadmium in the present study. It is enough to point out that in the embryonic gut, where cadmium (and also mercury) was strongly accumulated and retained, zinc also showed a high uptake. The embryonic gut thus may possibly be the main site both for the induction of teratogenic effects of cadmium (and mercury?) and for the antagonistic actions of zinc.

Another example of the essential role of zinc in embryogenesis

is the finding of Swenerton & Hurley (1971) that the teratogenic effects of ethylenediaminetetraacetic acid (EDTA) was prevented by zinc. Earlier the EDTA teratogenesis was ascribed to the calcium binding properties of this chelating agent (Tuchmann-Duplessis & Mercier-Parot 1956), but it seems now more probable that the zinc binding is the basis of the effects. There are thus many environmental pollutants that may interfere with human zinc metabolism and therefore also with embryonic development. In areas with a clinically recognizable human zinc deficiency (e.g. Egypt and Iran) there is a high rate of CNS malformations (Sever & Emanuel 1973; Sever 1975) of the type found in rats by Warkany & Petering (1972).

CHAPTER VII

GENERAL DISCUSSION

In almost all investigations that have been done on the placental passage of nutrients and chemicals the mature placenta has been studied. In review articles on the subject, very little information is found concerning the mode of transport and uptake into the early embryo. Still, when gross anatomical malformations are considered, the first four or five weeks of human pregnancy are most important. Not until day 22 of the pregnancy does a fetal circulation begin in the human placenta, and after that a continuous development occurs (Martin 1968). In the mouse the first signs of a fetal circulation in the chorioallantoic placenta (which corresponds to the human placenta) is seen at day 10-11 (Rugh 1968; Theiler 1972). Several drugs exert their teratogenic effect before this time. There is thus a lack of knowledge of how substances may be transferred to the early postimplantation embryo both in man and experimental animals. Since such information is difficult (if not impossible at the present stage) to get in man, this problem can so far only be studied in laboratory animals.

In this investigation rodents (mice, rats and golden hamsters) have been used. The main rationale of this choice is that most knowledge about teratogenic effects has been obtained by experimentation with these animals. Since the autoradiography technique used offers the possibilities of studying soluble substances as well as those probably more firmly bound to the embryonal or placental structures, it was considered appropriate to choose teratogens with very different chemical and pharmacological properties. These substances therefore have different effects on the embryo-fetus and their teratogenic phases generally do not correspond.

PATTERN OF UPTAKE THROUGHOUT GESTATION

Early gestation. From the results obtained in the present investigation it seems relevant to divide the whole gestational period - from the stage immediately preceeding organogenesis and up to birth - into two main periods: before and after the functional start of the chorioallantoic placenta. It has not been possible to determine the exact time from the literature, but it seems reasonable to believe

from the anatomical picture, and also e.g. from the results with 2,4,5-T (see chapter IV), that it is on the 10-11th day in the mouse. From this stage and subsequently a placental labyrinth and an allantoic circulation are present (Rugh 1968; Theiler 1972).

Before this stage of development the yolk sac must play an important role in the maternal-embryonic relationship. This is specially arranged in rodents and forms a so-called *inverted yolk sac placenta* (fig. VII:1). The yolk sac can be compared to an empty ball where one of the halves has been pushed into the other. The embryo is situated in the so formed cavity and is therefore almost completely surrounded by both layers of the yolk sac. At the opening of this inverted ball the chorioallantoic placenta will subsequently develop (in the ectoplacental cone). The outer wall of the ball, the so-called parietal (distal) yolk sac endoderm, rests on the Reichert's membrane and the whole seems to be easily permeable to large molecules as judged from functional and ultrastructural studies (Brambell & Hemmings 1949; Merker & Villegas 1970) and to most chemicals as judged by the present study. When passing this barrier the substances enter the yolk sac cavity.

In the inner wall of the ball the endoderm develops into an intestine-like epithelium facing the yolk sac cavity. It is this visceral (proximal) yolk sac endoderm which is absorbing and accumulating many chemicals (e.g. cadmium, mercury and trypan blue in this investigation) and it is often called the visceral yolk sac placenta. As this becomes richly vascularized it probably plays a major role in the embryo's nutrition before the chorioallantoic placenta develops. It may however also protect the embryo from foreign chemicals as they are often accumulated there like substances absorbed by a filter.

As this visceral yolk sac placenta offers the only passage, it means that all nutrients to the early embryo must be transported through it. Both zinc (chapter VI) and iron (unpublished) were taken up in the embryo at this stage, and so were the nonphysiological salicylic acid (chapter V) and thiouracil (unpublished). On the other hand, cadmium, mercury, trypan blue and 2,4,5-T (chapters I-IV) were not taken up in any detectable amounts in the embryo except in the gut (see below). As the number of substances so far investigated is limited, it is still not possible to determine which characteristic of a molecule that favours or impedes its transport in the visceral yolk sac placenta. As will be seen in the following discussion, there is not always a parallelism between the transport of the different

substances in this placenta in the early embryonic stages and that in the chorioallantoic placenta in late gestation.

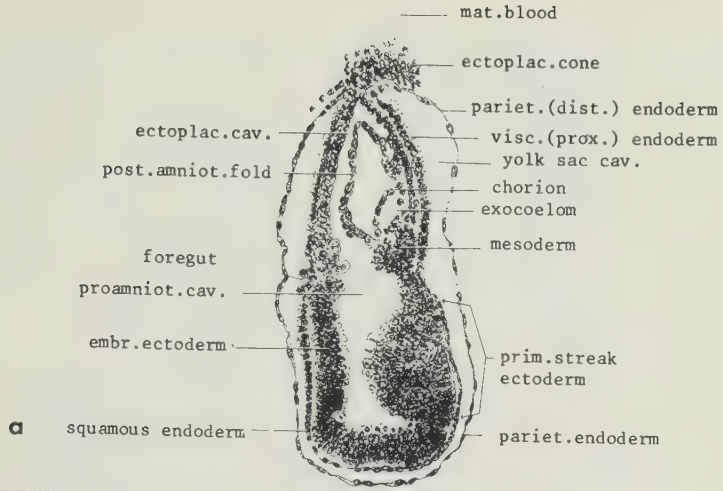
Not only the yolk sac placenta is derived from the visceral yolk sac endoderm, but also the gut of the embryo (figs VII:1 b and 2). From the beginning of organogenesis up to the moment when the vitelline duct closes* the yolk sac placental endoderm and the gut endoderm form a continuous layer. It means that substances which can pass into the yolk sac cavity and be absorbed by the yolk sac placenta (as e.g. cadmium, mercury and trypan blue in this investigation), may also be absorbed by the gut endoderm up to the time when the vitelline duct closes. This was found to be the case with these three substances. Upon the administration after the point of time indicated for the different animals (see footnote) no embryonic gut accumulation occurred. The significance of this finding will be discussed later.

Late gestation. The chorioallantoic placenta probably begins to function as a transport medium on day 10-11 in the mouse. After that a successive growth of the placenta occurs simultaneously with an attenuation of the layers separating the maternal and fetal circulations up to birth (Franke 1969, where these changes are described for the rat).

As the embryo-fetus increases its weight during the same time, the probable increase in permeability through the placental layers may not quite simply be considered as increasing the embryonic-fetal concentration of the different substances administered to the mother.

*The vitelline duct closes at about 9 1/2 days in the mouse (Rugh 1968), 9 - 9 1/4 days in the hamster (Boyer 1953) and 11 days in the rat (Biology Data Book 1972).

Fig. VII:1 (on the opposite page). Mouse embryos and surrounding membranes at (a) 7 days, (b) 8 days and (c) 9 days after conception. The yolk sac cavity is only a narrow space that almost completely surrounds the embryo. In (b) it can be seen that the embryonic endoderm is continuous with the visceral yolk sac. In (c) the stage is shown when the vitelline duct is closed. The maternal-fetal relationship seen in (c) will principally remain throughout gestation although there is a further development of the placental structure. At all stages of development the chemicals used in this study could be seen in the yolk sac cavity and sometimes they accumulated in the visceral yolk sac and gut endoderm. Other substances passed the endoderm and accumulated in all embryonic structures. (Modified after R. Rugh: The mouse. Its reproduction and development. Burgess Publ. Comp., Minneapolis, Minn., 1968. By courtesy of the publishers).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embr.ectoderm
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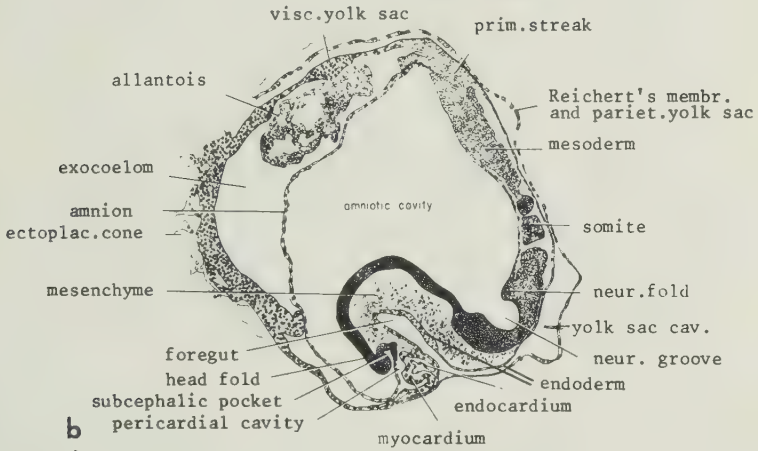
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head fold
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pericardial cavity
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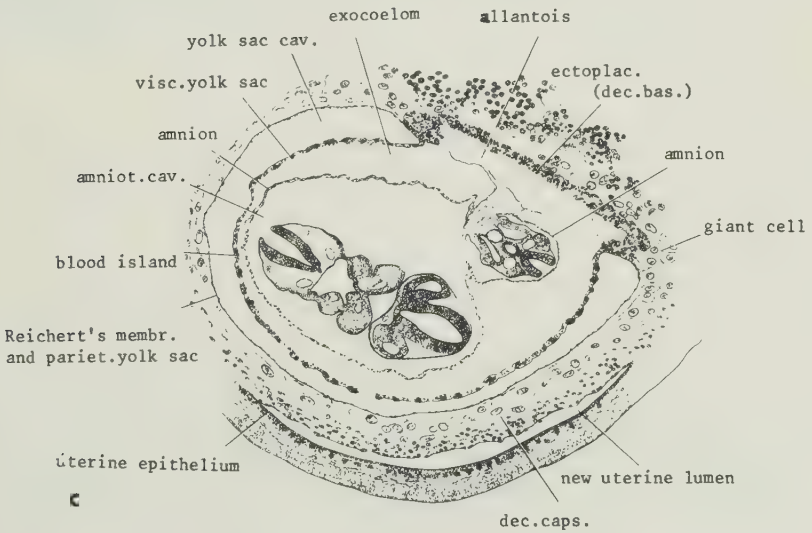
exocoelom
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giant cell
blood island
Reichert's membr. and pariet.yolk sac
uterine epithelium
new uterine lumen
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Fig. VII:2. Section through a mouse embryo and the surrounding membranes at 8 days 15 hours of gestation. At this stage different substances have been found to still pass into the gut lumen (through the vitelline duct) and accumulate in the gut endoderm. Some hours later this passage has disappeared as a route of transfer to the embryo. Hematoxylin-eosin (× 110).

In the literature contradictory informations on this point have been found (compare e.g. Schulert et al. 1969 and Mansour et al. 1974). However, the concentration of all substances which were measured quantitatively in the embryo-fetus from day 12 up to day 18 (inorganic mercury, 2,4,5-T and salicylic acid) showed a statistically significant increase with gestational age. In fig. VII:3 a the fetal tissue/maternal serum concentration ratios are compared for these three substances on different days of gestation. In fig. VII:3 b these values have been transferred to a semi-logarithmic diagram. In this case it can be seen that the regression line slopes for the respective substances differ and that the differences are statistically significant. Although the absolute increase was higher for salicylic acid than for mercury, its relative increase was significantly lower.

The visceral yolk sac placenta of the rodent seems to play a role in the transport throughout gestation and after day 10-11 one therefore has to consider two routes of transport to the fetus. It is uncertain whether the visceral yolk sac placenta changes its transport rate for each particular substance or if this rate can be considered constant throughout gestation (see chapter III, discussion). If we use the hypothesis that it is constant, then the chorioallantoic placenta must be held responsible for the increasing fetal concentration with advancing gestational age.

The results of the study of 2,4,5-T (chapter IV and fig. VII:3 a and b) most clearly follow the postulated model. Up to day 10-11 of gestation no 2,4,5-T was detected in the embryo (except in the gut), but after this time a steady increase in the fetal concentration was observed which paralleled, and was most probably the result of the maturation of the chorioallantoic placenta. The same was true for mercury (and a quaternary phenothiazine derivative [unpublished]) even if its passage was much lower and did not increase to such a degree. It is probable that the increasing fetal concentration of salicylic acid from day 12 to day 18 (fig. VII:3 a and b) was also a result of an increasing passage through the chorioallantoic placenta. In this case the relative increase was not very great, however, since there was also a high uptake in the embryo through the visceral yolk sac placenta (at least in early gestation).

Another interesting finding was the change in distribution within the embryo-fetus from early to late gestation. In the early stages both the essential zinc and drugs like salicylic acid and thiouracil (unpublished) were accumulated mostly in the neuroectoderm and in

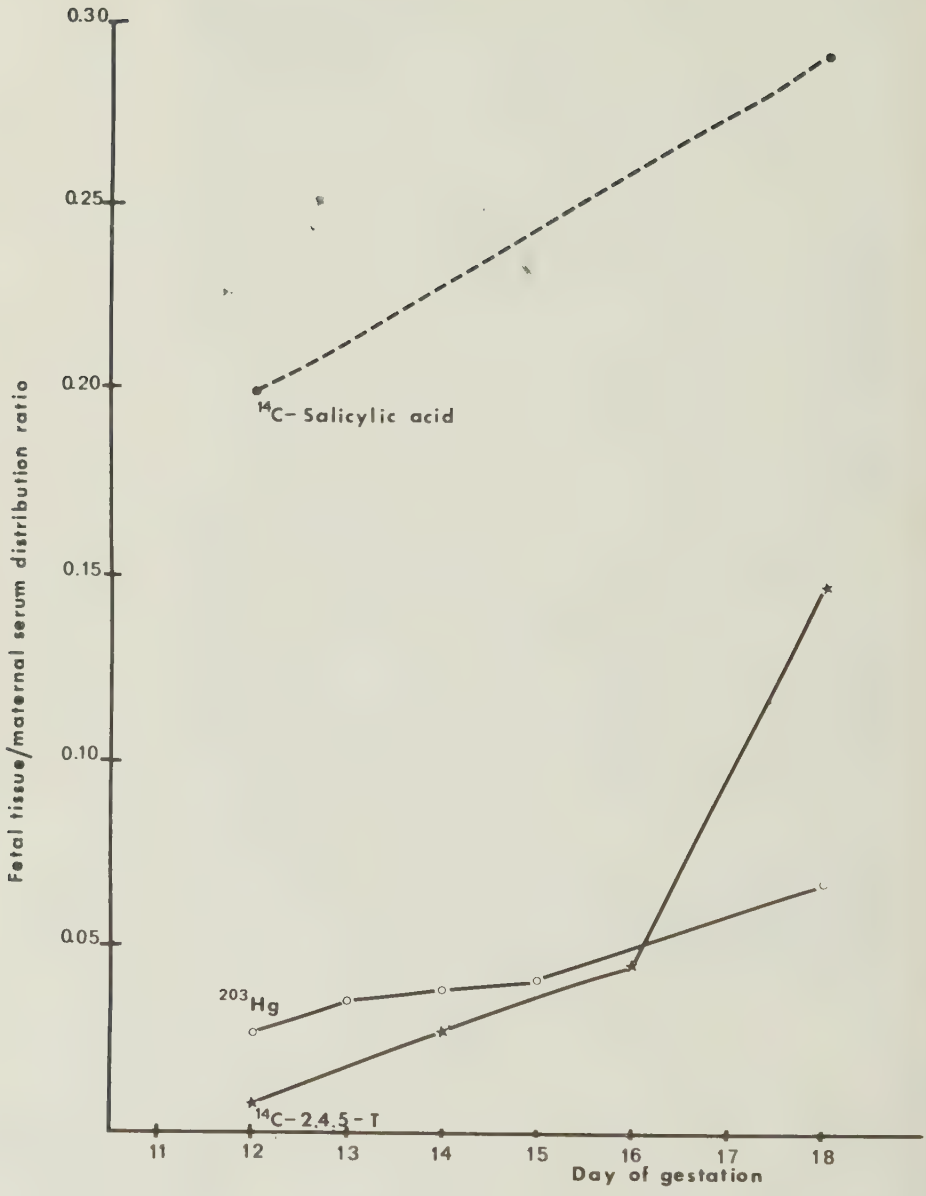


Fig. VII:3 a. Variations in radioactive distribution ratio of ^{14}C-salicylic acid, ^{14}C-2,4,5-T and ^{203}Hg (as $HgCl_2$) between homogenized fetuses (per gram tissue) and the maternal serum (per ml) as a function of gestational age. Salicylic acid and 2,4,5-T were injected 4 hr and mercury 8 hr prior to sampling on all the days indicated. All substances show an increased ratio with advancing gestational age, but this increase is most marked for 2,4,5-T. Each fetus was used as the sampling unit.

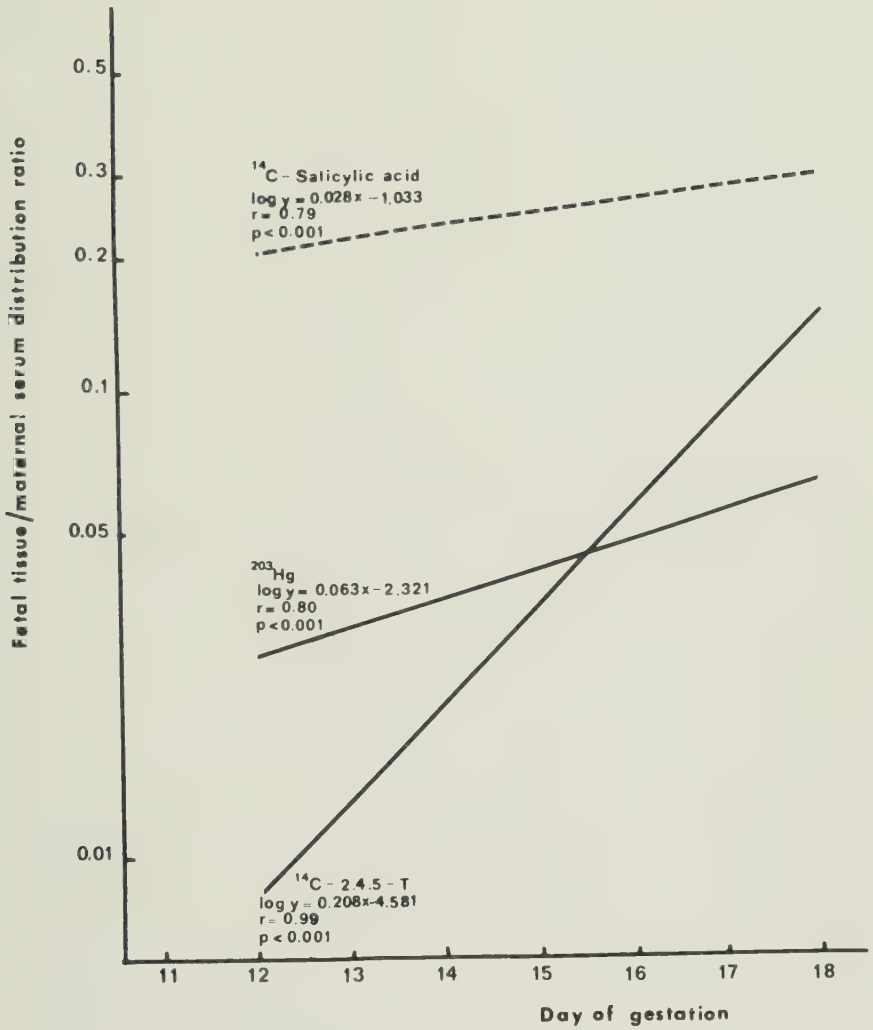


Fig. VII:3b. Regression lines according to the method of least squares for the three substances shown in fig. VII:3a after the y-axis has been transformed into a logarithmic scale. As in (a) each fetus was used as the sampling unit. The slopes for all three substances are statistically significant and the strength of the correlation is good. According to the student's t test the differences between each of the three slopes were highly significant ($p<0.001$).

other areas with rapid growth. It is possible that the accumulation in the neuroectoderm is part of the explanation why drugs (and also a zinc deficiency) often cause CNS malformations. On the other hand it has not generally been possible to show a specific accumulation of drugs in the organs reported to be damaged. The explanation may be that if there is a significant uptake in all the embryonic structures, only those organs which are in their most sensitive stage of development at the time of administration will be damaged. On the other hand, even when the embryonic organs are not very much differentiated, the various organs (or organ primordia) may naturally have individual biochemical characteristics that may explain the different degree of sensitivity to the different teratogens. Since embryonic development may be described as a long series of tissue interactions leading to the final structure it is possible that the damage to very different tissues may lead to a common final defect. Such a phenomenon would explain why chemically very different teratogens have a predilection for the same organ systems in their damage.

After organogenesis the drug distribution picture was often rather even, but at the end of gestation there was again a more differentiated distribution picture.* For the drugs investigated here, a blood-brain barrier was present in late gestation. Often an excretion pattern appeared, e.g. in the intestines and in the kidneys and the urinary bladder. At this stage substances with specific "target organs" in the adult will usually show affinity for the same organs in the fetus. On the whole the fetal distribution pattern became more similar to that of the maternal one with advancing gestation.

Placental structures. There was not always a parallelism between the visceral yolk sac and the chorioallantoic placental accumulation of chemicals. Cadmium and mercury were accumulated in both and were poorly transported through them. Trypan blue, which was heavily accumulated in the visceral yolk sac, could not be detected in the chorioallantoic placenta. Because this placenta is rich with blood a rather high concentration of the dye would not be detected due to quenching. Neither 2,4,5-T nor salicylic acid were accumulated in the chorioallantoic placenta. 2,4,5-T, but not salicylic acid, was accumulated in the visceral yolk sac (in late gestation). It was interesting to note that the cationic quaternary phenothiazine derivative (unpublished)

*Compare the series of illustrations in chapter V and read Introduction or Ullberg 1973.

(as well as the metal cations) was heavily accumulated in the chorio-allantoic placenta although transported less through it than were the organic acids.

Cadmium and mercury were localized in the steroid producing tissues of the ovary, which may be of importance for the progress of gestation and fetal well-being.

ACCUMULATION OF TERATOGENS RELATED TO EMBRYONIC-FETAL EFFECTS

On the whole the problem whether teratogens act directly on the embryo or via placental or maternal mechanisms may be considered unsolved. Influence on all these different "sites" may act together. It seems reasonable to believe that chemicals affecting cell division, replication of DNA or RNA and synthesis within the cell act on the embryonic and placental cells. Placental disturbances as a cause of fetal damage have been proposed for trypan blue (Beck et al. 1967) and both kidney (Brent et al. 1961) and yolk sac antibodies (Brent et al. 1971). Maternal influence was originally proposed as the mechanism of teratogenic action for trypan blue (Gillman et al. 1948). The present investigation has revealed both embryonic-fetal and placental sites of accumulation that may be related to damage to the embryo or fetus. My opinion is that intraembryonic mechanisms should primarily be considered. A summary is therefore made of the embryonic-fetal localizations found in this investigation and they are correlated to the type and period of damage of the embryo-fetus published by others.

In the literature on teratogenic action of chemicals it is striking that some agents act only in the early organogenetic period, some throughout this whole period and finally others only in the late organogenetic stage. In the first group, substances like cadmium (in hamsters), trypan blue and actinomycin can be found. In this investigation cadmium and trypan blue could be observed in the embryo (in the gut) when these substances were injected at the period of their teratogenic sensitivity, but not later. Actinomycin was found in a higher concentration in the early than in the late organogenetic stage in the mouse (all tissues) (unpublished) and in the rat (Jordan & Wilson 1970; Köhler & Merker 1972). This decrease in embryonic uptake of actinomycin also seems to coincide with an otherwise unexplainably early decrease of teratogenic sensitivity.

Schlüter (1970) showed that trypan blue caused a high rate of resorptions in mice when administered up to day 14 of gestation. The

teratogenic effect, however, rapidly decreased at day 9-10. The author's conclusion was that there may be different mechanisms for the teratogenic action versus the fetal death and resorptions. If the latter fetotoxic effects of trypan blue are mediated via placental structures, then also the chorioallantoic placenta may be affected after day 10-11. According to Schlüter this also means that there is still no explanation why the teratogenic activity ceases after day 10. My opinion is therefore that also for trypan blue an intraembryonic mechanism must be considered. As both trypan blue and cadmium are accumulated in the gut during the restricted time period when they are teratogenically active, but not later, this localization merits further attention.

Ferm et al. (1969) claimed that cadmium was taken up in the hamster embryo, which is apparently right, and that it was possibly localized in the developing visceral arch system (causing cleft lip and palate) which seems to be wrong. On the other hand, when the structures forming the oral and pharyngeal cavities are formed, it occurs while the stomoderm and the foregut are in intimate contact with each other. In early stages, before the mesoderm separates them, there is a close contact between the ectoderm and endoderm and later between the neuroepithelium and the gut. An accumulation of teratogens in the (gut) endoderm could explain a disturbed interaction between these layers (or organ anlagen respectively), causing e.g. exencephaly and spina bifida. It is however difficult to explain all the diverse teratogenic effects caused by cadmium and trypan blue by an accumulation in the gut, even if one takes into consideration the other interactions in organ formation. The transfer of undetectable amounts of these teratogens to the organs damaged remains as a possible explanation of the teratogenic effects by intrafetal mechanisms.

Salicylic acid belongs to the group of substances which causes congenital malformations or other fetotoxic effects (in mice and rats) irrespective of whether it is administered during the early or the late stages of organogenesis or in late gestation. Consistent with the present hypothesis of intraembryonic mechanisms salicylic acid was found to be taken up in the embryonic organs to a similar degree at all stages.

Cortisone is a teratogen that acts mainly during the late organogenetic period, causing a cleft palate. It seems that 2,4,5-T has similar characteristics, since the most important type of malformation it produces is a cleft palate, and this occurs most frequently when

2,4,5-T is administered during day 13-14. Even if a very high concentration is not reached in the embryos when 2,4,5-T is administered on these days, it may be high enough to cause an effect on palatal closure which seems to be the most sensitive process occurring at this stage. According to the same rationale the low teratogenic activity in the early organogenetic period may depend on the low (autoradiographically undetectable) concentration in the embryonic tissues at this time. It is questionable whether the gut accumulation of 2,4,5-T during the first days of organogenesis really is in the gut endoderm and not only in the lumen.

Even if there seems to be a positive correlation between intraembryonic accumulation and the ability to cause malformations, the role of the placental accumulation cannot be overlooked. Thus the cadmium, mercury or trypan blue accumulated in the visceral yolk sac placenta may act alone or together with the intraembryonically accumulated substance in producing the embryonic effect. The theory originally presented by Beck et al. (1967) concerning inhibited embryotrophic nutrition (produced by trypan blue) may still be valid in its main features even if the same authors recently changed their hypothesis considerably (Lloyd et al. 1974). Cadmium and mercury may disturb embryonic development by a similar mechanism. In late gestation the heavy accumulation of cadmium in the chorioallantoic placenta found in this study is probably the explanation for the severe necrosis of this structure - followed by fetal death reported by Pařizek (1964).

ZINC

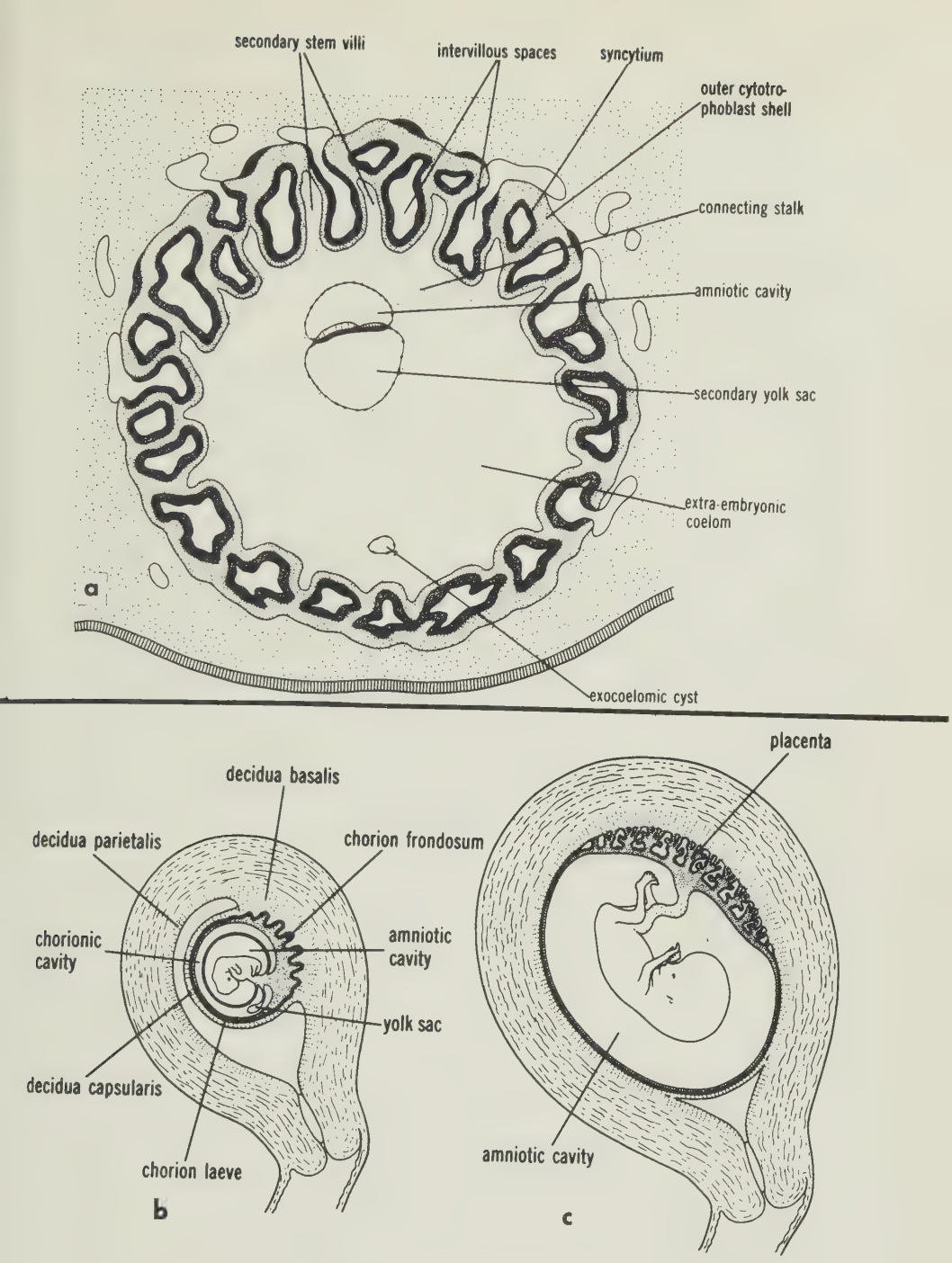
It has already been mentioned that zinc was taken up in both the early and late stages of gestation. The accumulation was relatively high especially in the ectodermal layer as early as one hr after the injection into a hamster at 8 days of gestation. Zinc was always found in high concentrations in the rapidly differentiating structures of the early embryo (e.g. neuroepithelium, endodermal structures, somites). This is another illustration of the essential role that is played by zinc in the embryonic development. A zinc deficiency is known to cause malformations (Hurley & Swenerton 1966) and decreased thymidine incorporation in the embryonic tissues (Swenerton et al. 1969). The teratogenic effects of cadmium (Ferm & Carpenter 1967) as well as EDTA (Swenerton & Hurley 1971) are counteracted by

zinc. Malformations of the CNS (anencephaly and spina bifida) occur more frequently in the geographical areas having a zinc deficiency than elsewhere (Sever 1975). The accumulation of zinc in the neuroepithelium of the early embryo is of interest when malformations are considered.

One often emerging question is whether the results obtained in animal experimental models are applicable to the situation in man. The mature chorioallantoic placenta of rodents and man are not very dissimilar when the layer separating the maternal and fetal blood are considered. Man has two trophoblast layers and the hamster, mouse and rat have three. In early gestation the morphological differences are more fundamental and it is difficult to evaluate their importance for drug passage to the embryo. The human fetus is not enveloped in such an epithelial sheet as is constituted by the visceral yolk sac in rodents. Here nutrients as well as foreign chemicals have to penetrate the decidua and the trophoblast layers (as in the rodents) and also a layer of extraembryonic mesoderm before they reach the exocoelomic cavity where the embryo (with its membranes) is floating (fig. VII:4). They finally have to pass either the amniotic membrane or the yolk sac membrane to reach the embryo. The yolk sac endoderm has absorptive characteristics (Hesseldahl & Falck Larsen 1969), but its role in transportation to the embryo is not clear. The amnion has been shown to be permeable to small molecules (Foreman & Segal 1973), but the nature of this characteristic in early pregnancy is not known.

It seems therefore more difficult to make a comparison between man and rodents concerning the transport of drugs and chemicals to the early embryo than it is to the late fetus. It is possible that the

Fig. VII:4 (on the opposite page). Development of embryo-fetus and surrounding membranes in man, (a) at the end of the 3rd week, (b) at the end of the 2nd month and (c) at the end of the 3rd month. In (a) the yolk sac is relatively large but is not surrounding the embryo, which leaves alternative ways of passage to the embryo (compare the mouse, fig. VII:1). In (b) the yolk sac is only a rather small vesicle and in (c) it is contained in the umbilical cord. (From J. Langman: Medical embryology, 2.ed. Williams & Wilkins, Baltimore, 1969. By courtesy of the publishers.)


secondary stem villi
intervillous spaces
syncytium
outer cytotrophoblast shell
connecting stalk
amniotic cavity
secondary yolk sac
extra-embryonic coelom
exocoelomic cyst
a
decidua basalis
decidua parietalis
chorion frondosum
chorionic cavity
amniotic cavity
yolk sac
decidua capsularis
chorion laeve
b
placenta
amniotic cavity
c

protective barrier constituted by the visceral yolk sac in rodents does not have any functional analogue in man, the embryo of which in that case would be reached also by substances blocked by the rodent visceral yolk sac. If substances are able to penetrate into the yolk sac cavity in man, then there are possibilities of a gut accumulation in the human embryo as in the rodents, which may be a risk for the embryo.

In conclusion it can be said that the degree of embryonic-fetal uptake changes throughout gestation, and that this change is different for various chemicals. The results show that for all teratogens studied, there is a detectable uptake in the embryo-fetus at the time of teratogenic sensitivity, although there is not generally an especially high accumulation in the organs to be most severely damaged. Also the opposite relationship seems to be valid for these teratogens - when there is no *teratogenic* sensitivity (see reference list) no prominent fetal uptake occurs. Fetal death and resorptions and growth retardation can be produced beyond these periods, however. These latter types of fetal damage as well as the teratogenic effects may, of course, wholly or partly be due to placental and/or maternal damage. Quantitatively the placental concentrations of teratogens are most often more prominent than the embryonic-fetal concentrations.

SUMMARY

A number of teratogens have been studied with regard to their distribution and tissue localization in pregnant rodents (mice, rats and golden hamsters). Special interest has been paid to the uptake in the embryo or fetus and the placental structures. The substances which have been selected are known to induce different effects on the embryo or fetus and they are also known to cause their damage during different stages in the fetal development.

The period from the presomite or early somite stages and up to the last days of gestation has been studied by means of whole body autoradiography and a modified technique - where the whole uteri of the animals have been removed, mounted in a horizontal plane and sectioned through all the embryos. The fetal concentrations have been quantitatively measured from day 12 to day 18 of gestation for some of the teratogens by impulse counting. During these days the weight of the fetuses, their dry weight percentage as well as their protein concentration have also been measured.

The heavy metals, cadmium ($^{109}CdCl_2$) and mercury ($^{203}HgCl_2$), and trypan blue (detected by its colour) accumulated in the visceral yolk sac endoderm and in the embryonic endoderm - which later forms the embryonic gut. This occurred up to the time of closure of the vitelline duct (about 9 days after conception in the mouse), which corresponds with the period of teratogenic effect of these substances. After this time none of the substances were detected in the embryo except mercury in late gestation.

The herbicide 2,4,5-T (^{14}C-2,4,5-T) did not pass to the embryonic tissues up to day 10-11 of gestation. During this early period the visceral yolk sac placenta is the only route to the embryo. From day 11 and up to day 18 a steadily increasing transport of 2,4,5-T to the fetal tissues occurred. This is the period of development and maturation of the chorioallantoic placenta. The results obtained with 2,4,5-T show that the visceral yolk sac and chorioallantoic placenta have different characteristics concerning the transport of comparatively small organic molecules. 2,4,5-T is a teratogen that produces defects only in the late organogenetic phase (day 13-14) in the mouse (cleft palate), when a significant concentration was found in the fetuses.

^{14}C-salicylic acid reached relatively high concentrations during the entire embryonic-fetal period which means that it is transported by both the visceral yolk sac and chorioallantoic placenta. In accordance with this, the salicylates are fetotoxic throughout gestation.

All the substances, the concentrations of which were measured quantitatively from day 12 to day 18 (mercury, 2,4,5-T and salicylic acid) showed an increasing fetal concentration with advancing stage of gestation, although it was exceptionally marked for 2,4,5-T (showing a 20-fold increase during this time).

Although these examples indicate a positive correlation between embryonic-fetal accumulation and the degree of damage, extraembryonic mechanisms must still be considered of importance in inducing fetal damage. Cadmium, mercury and trypan blue all accumulated in the placental structures throughout gestation, which may disturb the maternal-fetal relationship. The ovarian accumulation of cadmium may influence the progress of gestation.

Zinc ($^{65}ZnCl_2$), which has been shown to be essential for fetal development probably because of its involvement in DNA synthesis, accumulated in the most rapidly growing embryonic structures. In late gestation the fetal distribution pattern became more close to the maternal pattern. Zinc injected prior to gestation was transported to the embryos and fetuses and placental structures while only a minor amount of the cadmium injected before gestation was mobilized from the maternal organs.

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